DESCRIPTION

SCREENING METHOD FOR SOMATIC CELL NUCLEAR REPROGRAMMING SUBSTANCE

Technical Field

The present invention relates to a new screening method for a substance that reprograms somatic cell nucleus. More specifically, the present invention relates to a method of efficiently identifying a substance that induces the conversion of somatic cells to ES-like cells (a substance that induces somatic cell nuclear reprogramming) by monitoring the conversion to ES-like cells by the expression of a marker gene utilizing an ECAT gene. The present invention also relates to a method of efficiently selecting ES-like cells by monitoring the conversion to ES-like cells by the expression of a marker gene utilizing an ECAT gene. Furthermore, the present invention still also relates to a method of efficiently selecting a substance for the maintenance of undifferentiated state and pluripotency of ES cells by monitoring the maintenance of undifferentiated state and pluripotency of ES cells (maintenance of ES cell properties) by the expression of a marker gene utilizing an ECAT gene.

Background Art

Embryonic stem cells (ES cells) are stem cells established from an inner cell mass of mammalian blastocyst, and can be infinitely grown while maintaining their potential for differentiating into all types of cells (pluripotency). Focusing on this characteristic, there is expectation for stem cell therapy, which comprises treating a patient with myocardial infarction or Parkinson's disease by transplanting myocardial cells or nerve cells produced in large amounts from ES cells. However, ES cells involve the critical ethical issue of utilizing and sacrificing human fertilized eggs. On the other hand, tissue stem cells such as neural stem cells, hematopoietic stem cells, and mesenchymal stem cells are present in individual tissues of a living body. Tissue stem

cells do not involve the ethical issue because of the non-use of a fertilized egg, and avoid graft rejection because of the possible use of cells from the patient. However, tissue stem cells are difficult to isolate and the growth potential and differentiation potential thereof are much poorer than those of ES cells. If somatic cells such as tissue stem cells and differentiated cells can be converted to cells similar to ES cells having high growth potential and pluripotency by any means, the resulting ES-like cells would be ideal stem cells for clinical application. Specifically, it is hoped, for example, that somatic cells collected from a patient will be stimulated with a nuclear reprogramming factor (a factor for inducing nuclear reprogramming) to convert to ES-like cells, which ES-like cells will be clinically applied as stem cells. However, there is no system enabling the efficient search of such a nuclear reprogramming factor.

The term ECAT gene (ES cell associated transcript gene) generically refers to a series of genes specifically expressed in totipotent cells such as ES cells. A reported ECAT gene is the transcription factor Oct3 (also called Oct4 or POU5f1; hereinafter referred to as Oct3/4) gene. Although a similar gene has been reported in humans (hOct3/4 gene; Takeda et al., Nucleic Acids Research, 20:4613-4620 (1992)), there is no report of demonstrating the ES-cell-specific expression of the hOct-3/4 gene.

In recent years, our group has found nine genes specifically expressed in ES cells on the basis of computerized analysis utilizing an EST database and Northern blot analysis, and designating them as ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, the ECAT gene 6 gene, ECAT7 gene, ECAT8 gene, and ECAT9 gene (International Patent Publication No. WO 02/097090). Of these, ECAT4 is a factor also called Nanog, and has been shown to be an essential factor for the maintenance of the totipotency (pluripotency) of ES cells (Mitsui, K., et al., Cell, 113: 631-642 (2003)). ECAT5 is a factor also called ERas, and has been shown to promote the growth of ES cells

(Takahashi, K., et al., Nature, 423: 541-545 (2003)).

ECAT3 is a kind of F-box-containing protein, namely Fbx15, and is considered to be a ubiquitin ligase because it has the F-box. As a result of an analysis of the expression control region of the ECAT3 gene, ECAT3 was shown to undergo cooperative expression control by the two ES-cell-specific transcription factors Oct4 and Sox2 (Tokuzawa, Y., et al., Molecular and Cellular Biology, 23(8): 2699-2708 (2003)).

As a result of an analysis of a knock-in mouse resulting from knocking in β geo (the fusion gene of the β galactosidase and neomycin resistance genes) to the coding region of the ECAT3 gene, performed to examine the function of ECAT3, no evident abnormalities were observed in the mouse, nor was there any evident abnormality in the growth or differentiation potential of homozygous mutant ES cells. Based on this finding, the ECAT3 gene is considered not to be an essential factor for the maintenance and growth of ES cells (Tokuzawa, Y., et al., Molecular and Cellular Biology, 23(8): 2699-2708 (2003)).

Disclosure of the Invention

It is an object of the present invention to provide a system for efficiently selecting ES-like cells utilizing an ECAT gene, and a screening method for a somatic cell (tissue stem cell, differentiated cell) nuclear reprogramming substance utilizing the same system. It is another object of the present invention to provide a screening method for a substance for the maintenance of undifferentiated state and pluripotency of ES cells utilizing an ECAT gene.

As described above, if somatic cells can be converted to cells similar to ES cells having high growth poential and pluripotency by any means, the resulting ES-like cells would be ideal stem cells for clinical application. The present inventor diligently investigated in search for a method enabling efficient screening for a substance that induces such conversion to ES-like cells (somatic cell nuclear reprogramming

substance).

The present inventor first prepared somatic cells wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene. Specifically, somatic cells (lymphocytes) were prepared from a knock-in mouse wherein the β geo gene, which is a marker gene, was knocked in to the ECAT3 gene. These somatic cells were cultured under culture conditions for ES cell and selected using G418; all these cells died, with absolutely no drug resistant colony obtained. On the other hand, the aforementioned somatic cells were fused with normal ES cells, cultured under culture conditions for ES cell, and selected using G418; surviving cells emerged. As a result of an analysis of these surviving cells, they were found to express ECAT4 and Oct3/4, and hence to be ES-like cells having ES cell properties. From these experimental results, it was shown that the ES-like cells emerged due to the reprogramming of the nuclei of the somatic cells by fusion of the somatic cells and ES cells, and that the somatic cells became drug resistant due to the expression of β geo in place of the ECAT3 gene.

As described above, somatic cells comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of the ECAT3 gene express the marker gene only when converted to ES-like cells. Hence, it is possible to easily monitor the conversion to ES-like cells by the expression of a marker gene such as a drug resistance gene. Utilizing this property, a nuclear reprogramming factor that induces the conversion of somatic cells to ES-like cells can be efficiently screened with the expression of a marker gene such as a drug resistance gene as an index. Likewise, it is possible to efficiently select ES-like cells with the expression of the aforementioned marker gene as an index.

The present inventor and others further found that not only ECAT3 but also other ECATs such as ECAT2 and ECAT5 could be utilized for the aforementioned screening and selection of

ES-like cells. Because all ECAT genes (ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene) are known to be specifically expressed in ES cells, all ECATs can be used for the aforementioned screening. In particular, provided that an ECAT gene is destroyed by a technique such as knock-in, ECAT2 and ECAT3, which are not essential for the maintenance and growth of ES cells, are highly effectively utilized.

Furthermore, the aforementioned system for "easily monitoring the conversion to ES-like cells by the expression of a marker gene such as a drug resistance gene" can also be applied to screening for a substance for the maintenance of undifferentiated state and pluripotency of ES cells. Mouse ES cells permit the maintenance of undifferentiated state and pluripotency using a cytokine LIF. Furthermore, in the case of a large number of cells, mouse ES cells can be maintained using a serum-free medium supplemented with LIF without the use of feeder cells. However, at low densities, serum or feeder cells are essential. This indicates that an ES cell maintenance factor other than LIF is contained in serum and the secretion products of feeder cells. Although some of human ES cells can maintain their undifferentiated state and pluripotency on mouse feeder cells, not all cells can be maintained to remain undifferentiated. Furthermore, unlike in mouse ES cells, LIF is ineffective in human ES cells. This also suggests that feeder cells may secrete a factor for the maintenance of undifferentiated state and pluripotency of ES cells other than LIF, and also suggests the necessity of an additional factor differing from any secretion product of the feeder cells. When human ES cells are clinically applied, it is essential to culture them without the use of animal serum or feeder cells, and there is a demand for the identification of a factor for the maintenance of undifferentiated state and pluripotency of ES cells, but no method of efficient identification has been found.

According to the aforementioned system of the present

invention, it is possible to easily monitor the ES cell state by the expression of a marker gene such as a drug resistance gene; therefore, it is possible to easily screen for a substance (candidate) for the maintenance of undifferentiated state and pluripotency of ES cells by, for example, adding a test substance under culture conditions not allowing the maintenance of the ES cell state, and determining the presence or absence of cells expressing the marker gene.

The present invention was developed based on these findings.

Accordingly, the present invention provides the following:

(1) a screening method for a somatic cell nuclear reprogramming substance, which comprises the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene, and a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of the emergence of cells expressing the marker gene, and selecting a test substance allowing the emergence of the cells as a somatic cell nuclear reprogramming substance candidate,

(2) the screening method described in (1) above, wherein the ECAT gene is one or more genes selected from among the ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene,

(3) the screening method described in (1) or (2) above, wherein the marker gene is a drug resistance gene, a fluorescent protein gene, a luminescent enzyme gene, a chromogenic enzyme gene or a gene comprising a combination thereof,

(4) the screening method described in any of (1) to (3) above, wherein the somatic cell is a somatic cell comprising a gene resulting from knocking in the marker gene to the ECAT gene,

(5) the screening method described in (4) above, wherein the somatic cell is a somatic cell homozygously comprising the gene

resulting from knocking in the marker gene to the ECAT gene,

(6) the screening method described in (4) or (5) above, wherein the ECAT gene is one or more genes selected from among the ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene,

(7) the screening method described in (1) above, which comprises the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT2 gene, and a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the emergence of the surviving cells as a somatic cell nuclear reprogramming substance candidate,

(8) the screening method described in (1) above, which comprises the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT3 gene, and a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the emergence of the surviving cells as a somatic cell nuclear reprogramming substance candidate,

(9) the screening method described in (1) above, which comprises the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT5 gene, and a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the emergence of the surviving cells

emergence of the surviving cells as a somatic cell nuclear reprogramming substance candidate,

(10) the screening method described in (1) above, which comprises the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising genes resulting from knocking in a gene comprising a drug resistance gene to each of the ECAT2 gene and ECAT3 gene, and a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the emergence of the surviving cells as a somatic cell nuclear reprogramming substance candidate,

(11) the screening method described in (10) above, wherein the different drug resistance genes have been knocked in to ECAT2 gene and the ECAT3 gene,

(12) the screening method described in any of (7) to (11) above, wherein the somatic cell is a somatic cell homozygously comprising a gene resulting from knocking in a gene comprising a drug resistance gene to an ECAT gene,

(13) the screening method described in (1) above, which comprises the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT4 gene, and a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the emergence of the surviving cells as a somatic cell nuclear reprogramming substance candidate,

(14) the screening method described in (13) above, wherein the somatic cell is a somatic cell heterozygously comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT4 gene,

(15) the screening method described in (13) above, which

comprises the following steps (a) and (b):

(a) a step for supplying ECAT4 to a somatic cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT4 gene, and bringing it into contact with a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the emergence of the surviving cells as a somatic cell nuclear reprogramming substance candidate,

(16) the screening method described in (15) above, wherein the somatic cell is a somatic cell homozygously comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT4 gene,

(17) a nuclear reprogramming substance selected using the screening method described in any of (1) to (16) above,

(18) the nuclear reprogramming substance described in (17) above, which is a gene or protein derived from ES cells,

(19) the nuclear reprogramming substance described in (18) above, wherein the ES cell is an ES cell with the NAT1 gene destroyed,

(20) a substance derived from ES cells with the NAT1 gene destroyed,

(21) the substance described in (20) above, which is a cDNA library, a protein library, or a cell extract,

(22) a use of a knock-in mouse comprising a gene resulting from knocking in a marker gene to an ECAT gene as a source of the somatic cell used in the screening method described in any of (1) to (16) above,

(23) the use described in (22) above, wherein the knock-in mouse is a knock-in mouse homozygously comprising a gene resulting from knocking in a marker gene to an ECAT gene,

(24) the use described in (22) or (23) above, wherein the ECAT gene is one or more genes selected from among the ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene,

(25) the use described in any of (22) to (24) above, wherein the marker gene is a drug resistance gene, a fluorescent protein gene, a luminescent enzyme gene, a chromogenic enzyme gene or a gene comprising a combination thereof,

(26) a somatic cell comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene,

(27) the somatic cell described in (26) above, wherein the ECAT gene is one or more genes selected from among the ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene,

(28) the somatic cell described in (26) or (27) above, wherein the marker gene is a drug resistance gene, a fluorescent protein gene, a luminescent enzyme gene, a chromogenic enzyme gene or a gene comprising a combination thereof,

(29) the somatic cell described in any of (26) to (28) above, which comprises a gene resulting from knocking in a marker gene to an ECAT gene,

(30) the somatic cell described in (29) above, which homozygously comprises a gene resulting from knocking in a marker gene to an ECAT gene,

(31) the somatic cell described in (30) above, which is a differentiated ES cell homozygously comprising a gene resulting from knocking in a marker gene to the ECAT4 gene,

(32) the somatic cell described in (31) above, into which ECAT4 has been supplied,

(33) a selection method for ES-like cells, which comprises the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene, and a somatic cell nuclear reprogramming substance,

(b) a step following the aforementioned step (a), for selecting cells expressing the marker gene as ES-like cells,

(34) the selection method described in (33) above, wherein the

ECAT gene is one or more genes selected from among the ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene,

(35) the selection method described in (33) or (34) above, wherein the marker gene is a drug resistance gene, a fluorescent protein gene, a luminescent enzyme gene, a chromogenic enzyme gene or a gene comprising a combination thereof,

(36) the selection method described in (33) above, which comprises the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a drug resistance gene is present at a position permitting expression control by the expression control region of the ECAT2 gene, and a somatic cell nuclear reprogramming substance,

(b) a step following the aforementioned step (a), for selecting surviving cells in a selection medium as ES-like cells,

(37) the selection method described in (33) above, which comprises the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a drug resistance gene is present at a position permitting expression control by the expression control region of the ECAT3 gene, and a somatic cell nuclear reprogramming substance,

(b) a step following the aforementioned step (a), for selecting surviving cells in a selection medium as ES-like cells,

(38) the selection method described in (33) above, which comprises the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a drug resistance gene is present at a position permitting expression control by the expression control region of the ECAT5 gene, and a somatic cell nuclear reprogramming substance,

(b) a step following the aforementioned step (a), for selecting surviving cells in a selection medium as ES-like cells,

(39) the selection method described in (33) above, which

comprises the following steps (a) and (b):
(a) a step for bringing into contact with each other a somatic cell comprising genes wherein a drug resistance gene is present at a position permitting expression control by the expression control region of each of the ECAT2 gene and the ECAT3 gene, and a somatic cell nuclear reprogramming substance,
(b) a step following the aforementioned step (a), for selecting surviving cells in a selection medium as ES-like cells,
(40) the selection method described in (39) above, wherein mutually different drug resistance genes are present at the positions permitting expression control by the expression control regions of the ECAT2 gene and the ECAT3 gene,
(41) the selection method described in (33) above, which comprises the following steps (a) and (b):
(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a drug resistance gene is present at a position permitting expression control by the expression control region of the ECAT4 gene, and a somatic cell nuclear reprogramming substance,
(b) a step following the aforementioned step (a), for selecting surviving cells in a selection medium as ES-like cells,
(42) the selection method described in any of (33) to (41) above, wherein the somatic cell is a somatic cell comprising a vector incorporating a marker gene at a position permitting expression control by the expression control region of an ECAT gene,
(43) the selection method described in (42) above, wherein the ECAT gene is one or more genes selected from among the ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene,
(44) a use of the somatic cell described in any of (26) to (32) above in the screening method described in any of (1) to (16) above or the selection method described in any of (33) to (43) above,
(45) a cell expressing the marker gene or surviving cell that has emerged in the screening method described in any of (1) to

(16) above, or an ES-like cells selected in the selection method described in any of (33) to (43) above,

(46) a screening method for a substance for the maintenance of undifferentiated state and pluripotency of ES cells, which comprises the following steps (a) and (b):

(a) a step for bringing an ES cell comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene into contact with a test substance in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells,

(b) a step following the aforementioned step (a), for determining the presence or absence of cells expressing the marker gene, and selecting a test substance allowing the occurrence of the cells as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells,

(47) the screening method described in (46) above, wherein the ECAT gene is one or more genes selected from among the ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene,

(48) the screening method described in (46) or (47) above, wherein the marker gene is a drug resistance gene, a fluorescent protein gene, a luminescent enzyme gene, a chromogenic enzyme gene or a gene comprising a combination thereof,

(49) the screening method described in any of (46) to (48) above, wherein the ES cell is an ES cell comprising a gene resulting from knocking in a marker gene to an ECAT gene,

(50) the screening method described in (49) above, wherein the ES cell is an ES cell homozygously comprising a gene resulting from knocking in a marker gene to an ECAT gene,

(51) the screening method described in (49) or (50) above, wherein the ECAT gene is one or more genes selected from among the ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene,

(52) the screening method described in (46) above, which comprises the following steps (a) and (b):
(a) a step for bringing an ES cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT2 gene into contact with a test substance in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells,
(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the occurrence of the surviving cells as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells,
(53) the screening method described in (46) above, which comprises the following steps (a) and (b):
(a) a step for bringing an ES cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT3 gene into contact with a test substance in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells,
(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the occurrence of the surviving cells as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells,
(54) the screening method described in (46) above, which comprises the following steps (a) and (b):
(a) a step for bringing an ES cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT5 gene into contact with a test substance in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells,
(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the

occurrence of the surviving cells as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells,

(55) the screening method described in (46) above, which comprises the following steps (a) and (b):

(a) a step for bringing an ES cell comprising genes resulting from knocking in a gene comprising a drug resistance gene to each of the ECAT2 gene and the ECAT3 gene into contact with a test substance in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the occurrence of the surviving cells as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells,

(56) the screening method described in (55) above, wherein the different drug resistance genes have been knocked in to ECAT2 gene and the ECAT3 gene,

(57) the screening method described in any of (52) to (56) above, wherein the ES cell is an ES cell homozygously comprising a gene resulting from knocking in a gene comprising a drug resistance gene to an ECAT gene,

(58) the screening method described in (46) above, which comprises the following steps (a) and (b):

(a) a step for bringing an ES cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT4 gene into contact with a test substance in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the occurrence of the surviving cells as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells,

(59) the screening method described in (58) above, wherein the ES cell is an ES cell heterozygously comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT4 gene,

(60) a substance for the maintenance of undifferentiated state and pluripotency of ES cells selected using the screening method described in any of (46) to (59) above,

(61) the substance for the maintenance of undifferentiated state and pluripotency of ES cells described in (60) above, which is a secretion product of feeder cells,

(62) the substance for the maintenance of undifferentiated state and pluripotency of ES cells described in (60) above, which is a serum-derived component,

(63) a use of a knock-in mouse comprising a gene resulting from knocking in a marker gene to an ECAT gene as a source of the ES cell used in the screening method described in any of (46) to (59) above,

(64) the use described in (63) above, wherein the knock-in mouse is a knock-in mouse homozygously comprising a gene resulting from knocking in a marker gene to an ECAT gene,

(65) the use described in (63) or (64) above, wherein the ECAT gene is one or more genes selected from among the ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene,

(66) the use described in any of (63) to (65) above, wherein the marker gene is a drug resistance gene, a fluorescent protein gene, a luminescent enzyme gene, a chromogenic enzyme gene or a gene comprising a combination thereof,

(67) an ES cell comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene,

(68) the ES cell described in (67) above, wherein the ECAT gene is one or more genes selected from among the ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene,

(69) the ES cell described in (67) or (68) above, wherein the

marker gene is a drug resistance gene, a fluorescent protein gene, a luminescent enzyme gene, a chromogenic enzyme gene or a gene comprising a combination thereof,

(70) the ES cell described in any of (67) to (69) above, which comprises a gene resulting from knocking in a marker gene to an ECAT gene,

(71) the ES cell described in (70) above, which homozygously comprises a gene resulting from knocking in a marker gene to an ECAT gene, and

(72) a use of the ES cell described in any of (67) to (71) above in the screening method described in any of (46) to (59) above.

Brief Description of the Drawings

Figure 1 is a drawing showing an outline of Example 1. It is shown that fusion of lymphocytes isolated from an ECAT3$^{\beta geo/\beta geo}$ mouse and normal ES cells, and selection with G418 resulted in the emergence of ES-like cells positive for Oct3/4 and Nanog (ECAT4).

Figure 2 is a drawing showing the results of an analysis by flow cytometry (FACS) of cells selected with G418 from among cells resulting from fusion of lymphocytes isolated from an ECAT3$^{\beta geo/\beta geo}$ mouse and normal ES cells. It is shown that the size (FSC) about doubled and the DNA content (PI) quadrupled in the fusion cells (Fusion in the figure) compared with the pre-fusion cells (WT in the figure).

Figure 3 is a drawing showing the results of an analysis by RT-PCR of the expression of the ECAT2 gene in various cells and tissues. (A) shows the results from 25 repeated cycles of amplification by RT-PCR; (B) shows the results from 30 repeated cycles. ESG1 shows the results for ECAT2; NAT1 shows the results for the positive control NAT1. The individual lanes show the expression of ECAT2 or NAT1 in the following cells and tissues: lane 1: undifferentiated MG1.19 cells, lane 2: differentiated MG1.19 cells, lane 3: RT-MG1.19 cells, lane 4: undifferentiated RF-8 cells, lane 5: differentiated RF-8 cells,

lane 6: RT-RF-8 cells, lane 7: brain, lane 8: heart, lane 9: kidney, lane 10: testis, lane 11: spleen, lane 12: muscle, lane 13: lung, lane 14: stomach, lane 15: ovary, lane 16: thymus, lane 17: liver, lane 18: skin, lane 19: small intestine.

Figure 4 is a drawing showing the targeting vector for knocking in β geo (the fusion gene of the β galactosidase and neomycin resistance genes) or Hygro (hygromycin resistance gene) to the ECAT2 gene, and the concept of destruction of the ECAT2 gene using it.

Figure 5 is a drawing of a Southern blot analysis confirming the accurate occurrence of homologous recombination in the drug-resistant cells obtained by introducing a targeting vector into ES cells. In the figure, WT shows the results for ES cells not incorporating the vector. In the figure, -/- (lane Nos. 27, 35, and 36) shows the results for ECAT2 gene homozygous mutant ES cells wherein homologous recombination has occurred with both the β geo vector and the Hygro vector; β-geo +/- (lane Nos. 78, 30, 32, and 33) shows the results for ECAT2 gene heterozygous mutant ES cells wherein homologous recombination has occurred with the β geo vector; hygro +/- (lanes 4, 7, 31, and 34) shows the results for ECAT2 gene heterozygous mutant ES cells wherein homologous recombination has occurred with the Hygro vector.

Figure 6 is a drawing of a Northern blot analysis confirming the disappearance of the expression of the ECAT2 gene in ECAT2 gene homozygous mutant ES cells undergoing homologous recombination both with the β geo vector and with the Hygro vector. In the figure, the captions for the individual lanes are the same as Figure 5. The upper panel is an autoradiogram showing the results of a Northern blot analysis; the lower panel shows a photograph of ribosomal RNA stained with ethidium bromide.

Figure 7 is a drawing showing the results of an analysis of the fusion efficiency for normal ES cells (RF8) and thymocytes using a flow cytometer. Thymocytes derived from a mouse expressing a green fluorescent protein (EGFP) in the

whole body (CAG-EGFP mouse) and normal ES cells were fused under two conditions involving DC 300 V and 500 V ($RF8/T^{CAG-EGFP}$ in the figure); on the following day, the ratio of cells becoming EGFP-positive due to the fusion was determined using a flow cytometer.

Figure 8 is a drawing showing the results of an analysis of the fusion efficiency for NAT1 gene knockout ES cells and thymocytes using a flow cytometer. Using NAT1 gene knockout ES cells ($NAT1^{-/-}$(neo/Cre); previously deprived of the neomycin resistance gene), experiments similar to those of Figure 7 were performed.

Figure 9 is a graph showing the results of determinations of the nuclear reprogramming activities of normal ES cells and NAT1 gene knockout ES cells. Experiments of fusion of normal ES cells or NAT1 gene knockout ES cells and thymocytes derived from an ECAT3 knock-in mouse ($Fbx15^{-/-}$) were performed using various pulsation voltages. The number of ES-cell-like colonies emerging after selection with G418 was determined. The upper panel ($RF8/T^{Fbx15-/-}$) shows the results of a fusion experiment of RF8 and thymocytes derived from an ECAT3 knock-in mouse ($Fbx15^{-/-}$); the lower panel ($NAT1^{-/-}$(neo/Cre)/$T^{Fbx15-/-}$) shows the results of a fusion experiment of NAT1 gene knockout ES cells and thymocytes derived from an ECAT3 knock-in mouse ($Fbx15^{-/-}$). In the figure, the abscissa indicates pulsation voltage (V), and the ordinate indicates the number of ES-cell-like colonies emerging after selection with G418.

Best Mode for Embodying the Invention

Abbreviations for amino acids, (poly)peptides, (poly)nucleotides and the like used in the present description are based on the IUPAC-IUB rules [IUPAC-IUB Communication on Biological Nomenclature, Eur. J. Biochem., 138:9 (1984)], "Guideline for the Preparation of Descriptions etc. Including Base Sequences or Amino Acid Sequences" (edited by the Japan Patent Office), or abbreviations in common use in relevant fields.

"The ECAT gene (ES cell associated transcript gene)" as used herein generically refers to a series of genes specifically expressed in totipotent cells such as ES cells. Specifically, the ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene, and Oct3/4 gene can be mentioned (International Patent Publication No. WO 02/097090). The term ECAT gene as used herein sometimes refers not only to the cDNA (mRNA) of ECAT, but also to the genomic DNA of ECAT, depending on the technical contents.

The mouse and human types of base sequences and amino acid sequences of these ECAT cDNAs are described in International Patent Publication No. WO 02/097090. They are shown by the following sequence identification numbers in the sequence listing of the present description.

Table 1

ECAT gene	Mouse type base sequence	Mouse type amino acid sequence	Human type base sequence	Human type amino acid sequence
ECAT1	SEQ ID NO:1	SEQ ID NO:2	SEQ ID NO:3	SEQ ID NO:4
ECAT2	SEQ ID NO:5	SEQ ID NO:6	SEQ ID NO:7	SEQ ID NO:8
ECAT3	SEQ ID NO:9	SEQ ID NO:10	SEQ ID NO:11	SEQ ID NO:12
ECAT4	SEQ ID NO:13	SEQ ID NO:14	SEQ ID NO:15	SEQ ID NO:16
ECAT5	SEQ ID NO:17	SEQ ID NO:18	SEQ ID NO:19	SEQ ID NO:20
ECAT6	SEQ ID NO:21	SEQ ID NO:22		
ECAT7	SEQ ID NO:23	SEQ ID NO:24	SEQ ID NO:25	SEQ ID NO:26
ECAT8	SEQ ID NO:27	SEQ ID NO:28	SEQ ID NO:29	SEQ ID NO:30
ECAT9	SEQ ID NO:31	SEQ ID NO:32	SEQ ID NO:33	SEQ ID NO:34
Oct3/4	SEQ ID NO:35	SEQ ID NO:36	SEQ ID NO:37	SEQ ID NO:38

In the category of "ECAT genes" (ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene), not only the genes comprising any of the base sequences shown by the aforementioned sequence identification numbers, but also the genes comprising any base sequence similar to these base sequences, are included, as long as they are specifically

expressed in ES cells.

"The gene comprising a similar base sequence" as used herein refers to a gene comprising a base sequence resulting from the deletion, substitution or addition of one or more bases in any of the base sequences shown by the aforementioned sequence identification numbers and a gene comprising a base sequence having a high homology to any of the base sequences shown by the aforementioned sequence identification numbers can be mentioned.

"A gene comprising a base sequence having a high homology" as used herein means a gene that hybridizes with each ECAT gene under stringent conditions; specifically, a gene comprising a base sequence having a homology of 70% or more, preferably 80% or more, more preferably 90% or more, and particularly preferably 95% or more, to the base sequence shown by any of the aforementioned sequence identification numbers can be mentioned. Stringent conditions as mentioned herein can be adjusted by changing the temperatures, salt concentrations and the like during the hybridization reaction and washing as appropriate, and are set according to desired homology; for example, conditions involving a salt concentration of 6xSSC and a temperature of 65°C can be mentioned.

In the category of "ECAT" (ECAT1, ECAT2, ECAT3, ECAT4, ECAT5, ECAT6, ECAT7, ECAT8, ECAT9 and Oct3/4), not only the proteins comprising any of the amino acid sequences shown by the aforementioned sequence identification numbers, but also the proteins comprising any amino acid sequence similar to these amino acid sequences, are included, as long as they are specifically expressed in ES cells.

"A protein comprising a similar amino acid sequence" as mentioned herein refers to a protein encoded by a gene comprising the aforementioned similar base sequence.

The screening method of the present invention is a method of efficiently identifying a somatic cell nuclear reprogramming substance (a substance that induces the conversion to ES-like cells) by using somatic cells wherein a marker gene is present

at a position permitting expression control by the expression control region of an ECAT gene as the cells for screening, bringing a test substance into contact with the cells, and monitoring the conversion of the somatic cells to ES-like cells by the presence or absence of the emergence of cells expressing the marker gene. The present method is specifically described below.

(1) The screening method of the present invention for somatic cell nuclear reprogramming substance

The present invention provides a screening method for a somatic cell nuclear reprogramming substance, which comprises the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene and a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of the emergence of cells expressing the marker gene, and selecting a test substance allowing the emergence of the cells as a somatic cell nuclear reprogramming substance candidate,

Specifically, as the aforementioned "ECAT gene", one or more genes selected from among the ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene can be mentioned. The "one or more" as mentioned herein specifically refers to one or a combination of two to three ECAT genes, with preference given to one ECAT gene or a combination of two ECAT genes. Specifically, the ECAT2 gene, the ECAT3 gene, or a combination of the ECAT2 gene and the ECAT3 gene can be mentioned as examples.

Although the aforementioned ECAT gene may be an ECAT gene derived from any species such as mouse, rat, human, or monkey, with preference given to an ECAT gene derived from mouse or human.

The aforementioned "marker gene" refers to any gene that

enables cell sorting and selection by introducing the marker gene into cells. Specifically, a drug resistance gene, a fluorescent protein gene, a luminescent enzyme gene, a chromogenic enzyme gene or a gene comprising a combination thereof can be mentioned.

Specifically, as the drug resistance gene, the neomycin resistance gene (neo), tetracycline resistance gene (tet), kanamycin resistance gene, zeocin resistance gene (zeo), hygromycin resistance gene (hygro) and the like can be mentioned. When cells are cultured using a medium comprising each drug (referred to as a selection medium), only those cells incorporating and expressing the drug resistance gene survive. Therefore, by culturing cells using a selection medium, it is possible to easily select cells comprising a drug resistance gene.

Specifically, as the fluorescent protein gene, the GFP (green fluorescent protein) gene, YFP (yellow fluorescent protein) gene, RFP (red fluorescent protein) gene, aequorin gene and the like can be mentioned. Cells expressing these fluorescent protein genes can be detected using a fluorescence microscope. The cells can also be selected by separation and selection using a cell sorter and the like on the basis of differences in fluorescence intensity, or by subjecting the cells to limiting dilution to obtain a cell density of not more than one cell per well, then culturing and growing the cells, and detecting cells (wells) producing fluorescence under a fluorescence microscope. Furthermore, it is also possible to allow colonies to form on a soft agar medium and the like, and to select colonies under a fluorescence microscope and the like.

Specifically, as the luminescent enzyme gene, the luciferase gene and the like can be mentioned. Cells expressing these luminescent enzyme genes can be detected by measuring the amount of luminescence using a luminescence photometer with the addition of a luminescent substrate. The cells can also be selected by subjecting the cells to limiting

dilution to obtain a cell density of not more than one cell per well, then culturing and growing the cells, collecting a portion of the cells from each well, and measuring the presence or absence of luminescence with the addition of a luminescent substrate using a luminescence photometer.

Specifically, as the chromogenic enzyme gene, the β galactosidase gene, β glucuronidase gene, alkaline phosphatase gene, or secreted alkaline phosphatase SEAP gene and the like can be mentioned. Cells expressing these chromogenic enzyme genes can be detected by examining for chromogenic in the presence of a chromogenic substrate. The cells can also be selected by subjecting the cells to limiting dilution to obtain a cell density of not more than one cell per well, then culturing and growing the cells, collecting a portion of the cells from each well, and adding a chromogenic substrate to examine for chromogenic.

Specifically, as the gene comprising a combination of these marker genes, the β geo gene, which is the fusion gene of the neomycin resistance gene (neo) and the β galactosidase gene (β-gal), can be mentioned.

All the above-described marker genes are well known to those skilled in the art; vectors harboring such a marker gene are commercially available from Invitrogen, Inc., Amersham Biosciences, Inc., Promega, Inc., MBL (Medical & Biological Laboratories Co., Ltd.) and the like.

Of the aforementioned marker genes, a drug resistance gene or a gene comprising the drug resistance gene is particularly preferable because of the ease of cell selection.

"Somatic cells" as mentioned above means any cells except cells that maintain undifferentiated state and pluripotency, such as normal ES cells. Specifically, as examples, (1) tissue stem cells (somatic stem cells) such as neural stem cells, hematopoietic stem cells, mesenchymal stem cells, and spermatogonial stem cells, (2) tissue progenitor cells, (3) differentiated cells such as lymphocytes, epithelial cells, myocytes, and fibroblasts, (4) cells obtained by depriving ES

cells of their undifferentiated state and pluripotency by any technique, (5) cells that are fusion cells of somatic cells and ES cells, and that do not have an undifferentiated state and pluripotency, and the like can be mentioned.

"The ES-like cells" resulting from conversion of somatic cells by a nuclear reprogramming substance means cells having ES cell properties, that is, cells having undifferentiated state and pluripotency.

In the screening method of the present invention, somatic cells comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene are used as the cells for screening.

The "expression control region" as mentioned herein refers to a region for regulating the expression (transcription) of a gene, meaning a region comprising "a promoter region" or "promoter and enhancer regions".

There are various known methods of allowing a marker gene to be present at a position permitting expression control by the expression control region of an ECAT gene; the marker gene may be allowed to be present using any method well known to those skilled in the art. There are roughly two cases: (1-1) a case where a marker gene is allowed to be present utilizing an individual (mouse), and (1-2) a case where a marker gene is allowed to be present in a cellular level without utilizing an individual. A detailed description is given below.

(1-1) Method of allowing a marker gene to be present utilizing an individual (mouse)

When a marker gene is allowed to be present utilizing an individual (mouse), the marker gene is allowed to be present at a position on the genome for expression control by the expression control region of an ECAT gene. In this case, the ECAT gene present in the individual may be present in an expressible form, and may be present in a destroyed form.

The expression control region of a gene is normally present upstream of exon 1. Therefore, to ensure that a marker

gene undergoes expression control by the expression control region of an ECAT gene, it is desirable that the marker gene be present downstream of the exon 1 initiation site of the ECAT gene. In this case, the marker gene may be present at any position, as long as it is downstream of the exon 1 initiation site.

(1-1-a) Cases where the ECAT gene is destroyed

Although any method well known to those skilled in the art may be used to destroy the ECAT gene, the most commonly used technique comprises targeted-destroying the ECAT gene by homologous recombination using a vector that harbors a marker gene, and that causes homologous recombination at an optionally chosen position in the ECAT gene (hereinafter referred to as targeting vector), to allow the marker gene to be present instead at that position. Thus destroying an ECAT gene and allowing a marker gene to be present at that position is referred to as "knocking in a marker gene to an ECAT gene".

Although there are various known methods of so knocking in a marker gene, the promoter trap method is suitably used out of them. The promoter trap method comprises inserting a targeting vector not harboring a promoter into a genome by homologous recombination, and allowing the expression of a marker gene by an endogenous promoter (the ECAT gene promoter) if homologous recombination has occurred accurately. Specific examples of the method of allowing a marker gene to be present at a position permitting expression control by the expression control region of an ECAT gene by the promoter trap method are given below.

First, the genome sequence of an ECAT gene required for targeting is determined. The genome sequence can be sequenced utilizing already publicly known sequence information, if available in, for example, the public database Mouse Genome Resources (http://www.ncbi.nlm.nih.gov/genome/guide/mouse/) and the like. If no sequence information is available, by screening a genome library available to those skilled in the art by PCR and the like using one of the ECAT genes shown by

SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35 or 37 as a primer, it is possible to isolate a genomic clone comprising the genome region of desired ECAT gene, and to determine the genome base sequence. As examples of the genome library used here, the mouse BAC (bacterial artificial chromosome) library (Invitrogen), the PAC (P1-derived artificial chromosome) library (Invitrogen) and the like can be mentioned.

Next, on the basis of the genomic DNA sequence of the ECAT gene identified above, the genome region of the ECAT gene to be replaced by the marker gene is determined (hereinafter referred to as ECAT genome region A). The 5′-side region (5′-arm) and the 3′-side region (3′-arm) flanking this ECAT genome region A are amplified by performing PCR with genomic DNA as the template and the like. Here, as the genomic DNA serving as the template, the genomic DNA of a mouse BAC clone comprising an ECAT gene and the like can be mentioned. A primer for the PCR can be designed on the basis of the sequence of the aforementioned genomic DNA of the ECAT gene. The amplified 5′-arm and 3′-arm are inserted into respective sides flanking the marker gene cassette of the targeting vector for promoter trap. As examples of the targeting vector for promoter trap used here, pBSSK(-)-IRES-β geo, which comprises the IRES (internal ribosome entry site)-β geo (the fusion gene of the β galactosidase and neomycin resistance genes) cassette (Mountford P. et al., Proc. Natl. Sci. USA, 91:4303-4307 (1994)), a similar vector comprising the IRES-Hygro (hygromycin resistance gene) cassette and the like can be mentioned. Here, the IRES-Hygro cassette can be prepared by replacing the β geo portion of the aforementioned IRES-β geo cassette with Hygro (Invitrogen) and the like.

Next, the prepared targeting vector is linearized by digestion with restriction endonuclease, and this is introduced into ES cells by electroporation and the like.

As examples of the ES cells used for the introduction, ES cells such as RF8 cells (Meiner, V. et al., Proc. Natl. Acad.

Sci. USA, 93: 14041-14046 (1996)), JI cells (Li, E. et al., Cell, 69:915-926 (1992)), CGR8 cells (Nichols, J. et al., Development, 110:1341-1348 (1990)), MG1.19 cells (Gassmann, M. et al., Proc. Natl. Acad. Sci., USA, 92:1292-1296 (1995)), and commercially available mouse ES cells 129SV (No.R-CMTI-1-15, R-CMTI-1A), mouse ES cells C57/BL6 (No.R-CMTI-2A), and mouse ES cells DBA-1 (No.R-CMTI-3A) (all available from Dainippon Pharmaceutical Co., Ltd.) and the like can be mentioned.

Introduction of the targeting vector to ES cells is performed by electroporation (see Meiner, V. et al., Proc. Natl. Acad. Sci. USA, 93: 14041-14046 (1996) and the like), the calcium phosphate method, the DEAE-dextran method, the electroporation method, the method using a lipid for transfection (Lipofectamine, Lipofectin; Invitrogen) and the like. Subsequently, ES cells incorporating the targeting vector are selected on the basis of the characteristics of the marker gene used (for example, drug resistance gene). The accurate occurrence of homologous recombination in the ES cells selected can be confirmed by Southern blot using a portion of the ECAT gene as the probe and the like. Thus, ES cells heterozygously comprising a gene resulting from knocking in a marker gene to the ECAT gene on the genome can be prepared.

For culturing ES cells, any medium known to those skilled in the art may be used. In the case of RF8 cells, for example, a medium of the composition: 15% FBS, 0.1 mM Non Essential Amino Acids (GIBCO BRL), 2 mM L-glutamine, 50 U/ml penicillin-streptomycin, 0.11 mM 2-ME (GIBCO BRL)/Dulbecco's Modified Eagle Medium (DMEM), and the like can be mentioned. A commercially available prepared medium (for example, No.R-ES-101 from Dainippon Pharmaceutical Co., Ltd. and the like) can also be used.

When feeder cells are used in the cultivation of ES cells, the feeder cells used may be fibroblasts prepared from a mouse embryo by a conventional method or cells of an STO cell line derived from a fibroblast (Meiner, V. et al., Proc. Natl. Acad. Sci. USA, 93: 14041-14046 (1996)), and may be a

commercial product. As examples of the commercial product, feeder cells such as PMEF-N, PMEF-NL, PMEF-H, and PMEF-HL (all available from Dainippon Pharmaceutical Co., Ltd.) can be mentioned. It is desirable that the feeder cells be used for culturing the ES cells after their growth is stopped by mitomycin C treatment.

When the aforementioned feeder cells are not used in the cultivation of ES cells, the cultivation can be performed with the addition of an LIF (Leukemia Inhibitory Factor). As the LIF, mouse recombinant LIF, rat recombinant LIF (Nippon Chemi-Con Corporation and the like) and the like are used.

Next, ES cells comprising the aforementioned targeting vector are introduced into a mouse to prepare a knockout mouse (marker gene knock-in mouse). The method of preparing the marker gene knock-in mouse is well known to those skilled in the art. Specifically, a chimeric mouse is prepared by injecting the aforementioned ES cells to mouse (for example, C57BL/6 and the like) blastocysts, and transplanting the blastocysts into the uterus of a female mouse made to become pseudopregnant (ICR and the like). Subsequently, a heterozygous mutant mouse wherein a marker gene has been heterozygously knocked in is prepared by mating the chimeric mouse and an ordinary mouse (C57BL/6 and the like). By mating such heterozygous mutant mice, a homozygous mutant mouse wherein the marker gene has been homozygously knocked in is obtained.

The somatic cells used in the screening of the present invention may be somatic cells isolated from the aforementioned heterozygous mutant mouse, and may be somatic cells isolated from a homozygous mutant mouse. However, if an ECAT gene essential for the maintenance of the ES cell has been knocked out, it is necessary to use a somatic cell derived from a heterozygous mutant mouse in order to enable the step for converting somatic cells to ES-like cells and the maintenance of ES-like cells in the screening of the present invention. As an example of the ECAT gene essential for the maintenance of

the ES cell, the ECAT4 gene (Mitsui, K., et al., Cell, 113: 631-642 (2003)) can be mentioned. On the other hand, when an ECAT gene not essential for the maintenance of the ES cells is knocked out, a somatic cell derived from a heterozygous mutant mouse may be used, and a somatic cell derived from a homozygous mutant mouse may be used. As the ECAT gene not essential for the maintenance of the ES cell, the ECAT2 gene, the ECAT3 gene, and the ECAT5 gene can be mentioned. That is, as shown in the literature (Tokuzawa, Y., et al., Molecular and Cellular Biology, 23(8): 2699-2708 (2003)) for the ECAT3 gene, as shown in the literature (Takahashi, K., et al., Nature, 423: 541-545 (2003)) for the ECAT5 gene, and as demonstrated for the first time in an Example below for the ECAT2 gene, these ECATs are factors that do not influence the maintenance of ES cells. Of these ECATs, the ECAT2 gene and the ECAT3 gene do not influence not only the maintenance but also the growth of ES cells; therefore, when a somatic cell derived from a homozygous mutant mouse is used, it is preferable to utilize a somatic cell derived from a homozygous mutant knock-in mouse wherein a marker gene has been knocked in to the ECAT2 gene or the ECAT3 gene.

Because the marker gene expression level is doubled by homozygously comprising a gene resulting from knocking in a marker gene to an ECAT gene, compared with the heterozygous case, this is advantageous in that the selection of cells expressing the marker is made accurate and easy. From this viewpoint, the ECAT2 gene and the ECAT3 gene are very useful targets.

Furthermore, by mating homozygous mutant mice of different ECAT genes, a double knock-in mouse can be prepared. For example, by mating a homozygous mutant mouse of the ECAT2 gene and a homozygous mutant mouse of the ECAT3 gene, a double knock-in mouse wherein both the ECAT2 gene and the ECAT3 gene have been replaced with a marker gene can be prepared. In this case, it is preferable that mutually different marker genes have been knocked in to each ECAT gene. In this case, because

double selection with two different marker genes (for example, the neomycin resistance gene and the hygromycin resistance gene) is possible, the possibility of selecting false-positive ES-like cells in the screening of the present invention decreases, so that the likelihood of successful screening can be dramatically improved.

Specifically, somatic cells derived from a double knock-in mouse wherein the ECAT2 gene and the ECAT3 gene have been replaced with a marker gene, a double knock-in mouse wherein the ECAT2 gene and the ECAT4 gene have been replaced with a marker gene, a double knock-in mouse wherein the ECAT2 gene and the ECAT5 gene have been replaced with a marker gene, a double knock-in mouse wherein the ECAT3 gene and the ECAT4 gene have been replaced with a marker gene, a double knock-in mouse wherein the ECAT3 gene and the ECAT5 gene have been replaced with a marker gene, or a double knock-in mouse wherein the ECAT4 gene and the ECAT5 gene have been replaced with a marker gene, can be mentioned as examples. Preferably, a somatic cell derived from a double knock-in mouse wherein the ECAT2 gene and the ECAT3 gene have been homozygously replaced with a marker gene, can be mentioned.

(1-1-b) Cases where the ECAT gene is not destroyed

As the technique for allowing a marker gene to be present at a position permitting expression control by the expression control region of the ECAT gene without destroying the ECAT gene, a technique utilizing a transgenic non-human animal prepared by introducing the BAC vector or PAC vector, wherein a marker gene is present at a position permitting expression control by the expression control region of the ECAT gene, and the like to an individual such as a mouse or rat can be mentioned. A description is given below for the BAC vector.

The BAC clone comprising the expression control region of an ECAT gene used here can be isolated and identified on the basis of the sequence information on the ECAT gene, as stated in (1-1-a) above. Replacement of a portion of the ECAT gene with a marker gene in the BAC clone comprising the ECAT gene

can easily be performed using, for example, Red/ET Recombination (Gene Bridges). The expression control region of each ECAT gene is normally present upstream of the exon 1 of the ECAT gene. Therefore, to ensure that a marker gene undergoes expression control by the expression control region of the ECAT gene, it is desirable that the marker gene be present downstream of the exon 1 of the ECAT gene. In this case, the marker gene may be present at any position on the ECAT gene, as long as it is downstream of exon 1.

Methods of preparing a transgenic animal incorporating the thus-prepared BAC vector wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene (hereinafter also referred to as the BAC vector comprising a marker gene) are well known; the transgenic animal can be prepared on the basis of, for example, extra issue of Jikken Igaku "Shin Idenshi Kogaku Handbook, 3rd revised edition" (Yodosha Co., Ltd., 1999) and the like. A description of how to prepare a transgenic animal is given below for a mouse.

The method of introducing a gene into a mouse fertilized egg is not subject to limitation; the introduction is possible by the microinjection method, the electroporation method and the like. After the introduction, the egg obtained is cultured and transplanted to the oviduct of a pseudo-dam mouse, after which the recipient mouse is grown, and a desired pup mouse is selected from among the pup mice born. This selection can be performed by, for example, examining the DNA derived from the pup mouse for the presence or absence of the introduced gene by the dot blot hybridization method or the PCR method.

The aforementioned pup mouse and a wild mouse are mated to prepare a hetero-transgenic mouse (a mouse heterozygously comprising the introduced gene). By mating heterozygous mice, a transgenic mouse homozygously comprising the BAC vector comprising a marker gene can be obtained.

The somatic cells used in the screening of the present invention may be somatic cells isolated from the aforementioned

hetero-transgenic mouse, and may be somatic cells isolated from a homo-transgenic mouse. Because the ECAT gene itself is expressed in this transgenic mouse, unlike in the case of the aforementioned knock-in mouse, it is unnecessary to take into consideration whether or not the ECAT gene used is essential to the maintenance of ES cells. Therefore, the somatic cells can be equally used for all ECAT genes (ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene), and because the marker gene expression level is high, it is preferable to utilize a transgenic mouse homozygously comprising a marker gene.

Furthermore, a double transgenic mouse can be prepared by mating transgenic mice of different ECAT genes. In this case, the individual transgenic mice mated preferably comprise mutually different marker genes. In this case, because double selection with two different marker genes (for example, neomycin resistance gene and hygromycin resistance gene) is possible, the possibility of selecting false-positive ES-like cells in the screening of the present invention decreases, so that the likelihood of successful screening can be dramatically improved.

The somatic cells isolated from the above-described knock-in mouse or transgenic mouse may be any cells wherein the marker gene is not expressed (or is expressed at low expression levels). Specifically, cells other than totipotent cells such as ES cells can be mentioned; for example, (1) tissue stem cells (somatic stem cells) such as neural stem cells, hematopoietic stem cells, mesenchymal stem cells, and spermatogonial stem cells, (2) tissue progenitor cells, or (3) differentiated cells such as lymphocytes, epithelial cells, myocytes, fibroblasts can be mentioned. The cells can be isolated by a technique well known to those skilled in the art.

When ES cells have been isolated, they should be used after being deprived of their undifferentiated state and pluripotency by any technique (described below).

As described above, it becomes possible to easily prepare

somatic cells from any tissue by maintaining somatic cells wherein a marker gene has been knocked in to an ECAT gene, or somatic cells incorporating a marker gene, at individual (mouse) levels, the aforementioned technique is a highly preferable method of supplying somatic cells.

(1-2) Method of allowing a marker gene to be present at cellular levels without utilizing an individual

There are various known methods of allowing a marker gene to be present at a position permitting expression control by the expression control region of an ECAT gene in cells without utilizing an individual; the marker gene may be allowed to be present using any method well known to those skilled in the art. Generally, a method of introducing a vector harboring a marker gene into cells can be mentioned.

The cells used for the transfection may be somatic cells or ES cells. The somatic cells used here may be somatic cells derived from any species such as mouse, human, or monkey. The somatic cells may be primary culture cells or an established line of cells; specifically, primary culture cells such as mouse embryoric fibroblasts (MEF), bone marrow derived mesenchymal stem cells, or spermatogonial stem cells, and established lines of cells like NIH3T3 and the like can be mentioned. As the ES cells, human or simian ES cells, as well as the mouse ES cells mentioned above, can be used. Here, as the human ES cells, KhES-1, KhES-2 or KhES-3 (all available from Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University) and the like can be mentioned; as the simian ES cells, cynomolgus monkey ES cells (Asahi Techno Glass Corporation) can be mentioned. When these ES cells are used in the screening of the present invention, they should be used after being deprived of their undifferentiated state and pluripotency by any technique.

For vector introduction into cells, an ordinary method of introduction suitable to the aforementioned host cell may be used. Specifically, the calcium phosphate method, the DEAE-dextran method, the electroporation method, the method using a

lipid for transfection (Lipofectamine, Lipofectin; Invitrogen) and the like can be mentioned.

As the vector used for the introduction, the BAC vector and the PAC vector, which are vectors enabling cloning up to about 300-kb DNA, plasmid vectors, and the targeting vector described in (1-1) above and the like can be mentioned. Hereinafter described are methods of preparing a somatic cell wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene using each of these vectors.

(1-2-a) Cases where the BAC vector or the PAC vector is used

By utilizing the BAC vector or PAC vector comprising the expression control region of an ECAT gene, it is possible to allow a marker gene to be present at a position permitting expression control by the expression control region of the ECAT gene. A description is given below for the BAC vector.

The BAC clone comprising the expression control region of an ECAT gene used here (hereinafter referred to as the BAC clone comprising an ECAT gene) can be isolated and identified on the basis of the sequence information on the ECAT gene, as stated in (1-1) above. Replacement of a portion of the ECAT gene with a marker gene in the BAC clone comprising the ECAT gene can easily be performed using, for example, Red/ET Recombination (Gene Bridges). The expression control region of each ECAT gene is normally present upstream of the exon 1 of the ECAT gene. Therefore, to ensure that a marker gene undergoes expression control by the expression control region of the ECAT gene, it is desirable that the marker gene be present downstream of the exon 1 initiation site of the ECAT gene. In this case, the marker gene may be present at any position, as long as it is downstream of the exon 1 initiation site.

By introducing the thus-prepared BAC vector wherein a marker gene is present at a position permitting expression control by the expression control region of the ECAT gene to a somatic cell, the cell can be provided as a somatic cell for

the screening of the present invention. The BAC vector introduced here may be one kind of BAC vector, and may be two or more kinds of BAC vectors comprising different ECAT genes. To enable the easy selection of the cell incorporating the BAC vector in a selection medium, it is preferable that a gene comprising a drug resistance gene (hereinafter referred to as a second drug resistance gene) be inserted into the BAC vector. In this case, to enable the expression in the somatic cell, it is necessary that a promoter expressed in the somatic cell be added to the 5′ side or 3′ side of the second drug resistance gene. Although the second drug resistance gene may be the same kind of drug resistance gene as the marker gene present at a position permitting expression control by the expression control region of the ECAT gene, and may be a different kind of drug resistance gene, it is desirable that the second drug resistance gene be a different kind of drug resistance gene. When the same kind of drug resistance gene is used, it is possible to previously add the loxP sequence or FRT sequence to both ends of the second drug resistance gene, and select cells incorporating the BAC vector in a selection medium, and then cleaving out the second drug resistance gene with the recombinase Cre or FLP.

When a second drug resistance gene is not inserted into the BAC vector, unlike in the aforementioned case, a second expression vector harboring the second drug resistance gene may be co-transfected with the aforementioned BAC vector, and selection may be performed using a selection medium. In that case, it is desirable that the transfection be performed using the BAC vector in large excess compared with the second expression vector.

When the BAC vector wherein a marker gene is present at a position permitting expression control by the expression control region of the aforementioned ECAT gene has been introduced into ES cells, ES cells incorporating and expressing the marker gene can be selected on the basis of the properties of the marker gene used. Subsequently, by allowing the

differentiation of the ES cells into somatic cells, the ES cells can be converted to a somatic cell used for the screening of the present invention. Because ES cells differentiate during culturing conditions without feeder cells, somatic cells obtained by differentiation under these conditions and somatic cells obtained by differentiation using a differentiation inducer known to those skilled in the art, such as retinoic acid, can be used for the screening of the present invention. Here, as examples of the somatic cells differentiated from ES cells, tissue stem cells, tissue progenitor cells, or somatic cells (nerve cells, dermal corneal cells, myocardial cells, skeletal muscle cells, blood cells, islet cells or pigment cells and the like) can be mentioned.

(1-2-b) Cases where a promoter-free plasmid vector is used

By inserting the fusion gene of the expression control region of an ECAT gene and a marker gene into a promoter-free plasmid vector and transforming cells therewith, cells for the screening of the present invention can be prepared.

As examples of the vector used here, promoter-free plasmid vectors such as pBluescript (Stratagene) and pCR2.1 (Invitrogen) can be mentioned.

As examples of the expression control region of an ECAT gene used here, an about 1-kb portion, preferably an about 2-kb portion, upstream of the transcription initiation site of the gene can be mentioned.

The expression control region of each ECAT gene can be identified by, for example, a technique comprising (i) a step for determining the 5′ end by an ordinary method such as the 5′-RACE method (performed using, for example, the 5′ full Race Core Kit (manufactured by Takara Shuzo Co., Ltd.) and the like), the oligo cap method, or S1 primer mapping; and (ii) a step for acquiring a 5′-upstream region using the Genome Walker Kit (manufactured by CLONTECH Laboratories Japan, Ltd.) and the like, and determining the promoter activity of the upstream region obtained, and the like. By fusing a marker gene to the 3′ side of the thus-identified expression control region of the

ECAT gene, and inserting this into the aforementioned plasmid vector, a plasmid vector wherein the marker gene is present at a position for expression control by expression control region of the ECAT gene can be prepared.

By introducing the vector thus prepared into a somatic cell or ES cell in the same manner as (1-2-a) above, a somatic cell for the screening of the present invention can be prepared.

(1-2-c) Cases where a targeting vector is used

By introducing the targeting vector described in (1-1) above into a somatic cell or ES cell, a somatic cell for the screening of the present invention can also be prepared.

When the aforementioned targeting vector is introduced into a somatic cell, it is more preferable to use a somatic cell obtained by allowing a gene comprising a drug resistance gene (second drug resistance gene) to be present on the targeting vector in the same manner as (1-2-a) above, or co-transfecting a second expression vector comprising a second drug resistance gene with the targeting vector, in order to enable the easy selection of cells incorporating the vector in a selection medium, and selecting using a selection medium, for the screening of the present invention. In the latter case, it is desirable that the transfection be performed using the aforementioned targeting vector in large excess compared with the second expression vector.

The aforementioned somatic cell may heterozygously comprise a gene resulting from knocking in a marker gene to an ECAT gene, and may homozygously comprise the same. When the ECAT4 gene is utilized, it is desirable that the aforementioned knock-in gene be heterozygously comprised; when the same is homozygously comprised, ECAT4 may be supplied into the cell at the time of screening. When the ECAT2 gene, ECAT3 gene or ECAT5 gene (particularly the ECAT2 gene or ECAT3 gene) is utilized, it is desirable that the aforementioned knock-in gene be homozygously comprised. A somatic cell homozygously comprising a gene resulting from knocking in a marker gene to

an ECAT gene can be prepared by further introducing an additional knock-in gene (a targeting vector comprising a marker gene) into a somatic cell heterozygously comprising a knock-in gene (a targeting vector comprising a marker gene). The somatic cell can also be selected by culturing a somatic cell heterozygously comprising a knock-in gene in a selection medium comprising a high concentration of drug.

Furthermore, by introducing another knock-in gene (a gene wherein another ECAT gene has been knocked out) into a somatic cell homozygously comprising the aforementioned knock-in gene, a double knock-in cell similar to that obtained in (1-1) above can be prepared.

When the aforementioned targeting vector is introduced into ES cells, cells incorporating and expressing the marker gene can be selected on the basis of the properties of the marker gene on the targeting vector. The ES cells, like the aforementioned somatic cells, may also heterozygously comprise a gene resulting from knocking in a marker gene to an ECAT gene, and may also homozygously comprise the same. For the method of preparing homozygous mutant cells, refer to the method of preparing ECAT2 gene homozygous mutant ES cells described in Example 3 below. The method of inducing the conversion of ES cells to somatic cells is the same as (1-2-a) above.

As described in the literature (Mitsui, K., et al., Cell, 113: 631-642 (2003)) and WO 2004/067744), ES cells wherein the ECAT4 gene has been homozygously mutated (ES cells wherein a marker gene has been knocked in to the ECAT4 gene) are known to no longer maintain undifferentiated state and pluripotency, hence to have differentiated. Although this cell was infected with a retroviral vector harboring the ECAT4 gene and allowed to normally express ECAT4 therein, ES cell functions (undifferentiated state and pluripotency) have not been restored.

Because ECAT4 is an essential factor for the maintenance of ES cell functions (undifferentiated state and pluripotency),

cells that are ECAT4 homozygous mutant ES cells, to which ECAT4 has been supplied, can be said to be differentiated cells in a state similar to that of ES cells. Therefore, a screening system for bringing these cells into contact with a test substance is an efficient screening system enabling the easier identification of a nuclear reprogramming substance; the ECAT4 homozygous mutant ES cells used for such screening, and the cells to which ECAT4 has been supplied are somatic cells preferred for the present invention.

In the screening step (a) of the present invention, a somatic cell thus prepared and a test substance are brought into contact with each other.

The test substance (test sample) used here is not subject to limitation, and is exemplified by a nucleic acid, a peptide, a protein, an organic compound, an inorganic compound or a mixture thereof and the like; the screening of the present invention is specifically performed by bringing these test substances into contact with the aforementioned somatic cell. More specifically, as the test substance, a cell extract, a gene (genome, cDNA) library, an RNAi library, an antisense nucleic acid, a gene (genome, cDNA, mRNA), a protein, a peptide, a low molecular compound, a high molecular compound, a natural compound and the like can be mentioned. More specifically, the ES cell shown in Examples, egg, cell extract of ES cell or egg (extraction fraction), cDNA library, genome library or protein library derived from ES cells or egg, or growth factor and the like can be mentioned.

As a derivation for the cDNA library, protein library or cell extract (organic compound, inorganic compound and the like), undifferentiated cells such as ES cells or eggs are preferable, as described above, and ES cells wherein the NAT1 gene has been destroyed (knocked out) are particularly effective.

The NAT1 gene is a gene similar to the protein translation initiation factor eIF4G, and it has been reported that if the NAT1 gene is destroyed in ES cells, the

undifferentiated state is enhanced compared with the normal condition (Yamanaka, S. et al., Embo J., 19, 5533-5541 (2000)). However, no association with nuclear reprogramming has been shown.

As described in Examples below, the present inventor fused an NAT1 gene knockout ES cell and a thymocyte derived from an ECAT3 knock-in mouse, and performed selection with G418; the incidence of ES-cell-like colonies was much higher than that obtained using a normal ES cell. This shows that NAT1 gene knockout ES cells are higher than normal ES cells in terms of not only the degree of undifferentiated state, but also reprogramming activity, and are considered to be very effective as a derivation for the cDNA library and the like used for the screening of the present invention.

Here, a cDNA library can be constructed using a commercially available cDNA library construction kit (for example, CloneMinor cDNA library construction kit (Invitrogen) or Creator SMART cDNA library construction kit (BD Biosciences) and the like). A protein library can be constructed with reference to WO 00/71580 and the like.

Note that the aforementioned cDNA library, protein library or cell extract and the like derived from NAT1 gene knockout ES cells can be effectively used not only in the screening of the present invention, but also in any functional screening for a nuclear reprogramming factor.

These test substances are brought into contact with somatic cells in an embodiment incorporatable into the somatic cells. For example, when the test sample is a nucleic acid (cDNA library and the like), it is introduced into a somatic cell using calcium phosphate, DEAE-dextran, a lipid for transfection or electric pulse and the like.

The conditions of contact of a somatic cell and a test substance are not subject to limitation, as long as they are culturing conditions (temperature, pH, medium composition and the like) that do not kill the cell, and that are suitable for the incorporation of the test substance.

Cell culture is performed under culture conditions for ES cell before, at, or after, the aforementioned contact of a somatic cell and a test substance. The cultivation of ES cells may be performed using any method known to those skilled in the art. In the case of RF8 cells, for example, a medium of the composition: 15% FBS, 0.1 mM Non Essential Amino Acids (GIBCO BRL), 2 mM L-glutamine, 50 U/ml penicillin-streptomycin, 0.11 mM 2-ME (GIBCO BRL)/Dulbecco's Modified Eagle Medium (DMEM), and the like can be mentioned. A commercially available prepared medium (for example, No.R-ES-101 from Dainippon Pharmaceutical Co., Ltd. and the like) can also be used.

When feeder cells are used in the cultivation of ES cells, the feeder cells used may be fibroblasts prepared from a mouse embryo by a conventional method or cells of an STO cell line derived from a fibroblast (Meiner, V. et al., Proc. Natl. Acad. Sci. USA, 93: 14041-14046 (1996)), and may be a commercial product. As examples of the commercial product, feeder cells such as PMEF-N, PMEF-NL, PMEF-H, and PMEF-HL (all available from Dainippon Pharmaceutical Co., Ltd.) can be mentioned. It is desirable that the feeder cells be used for culturing the ES cells after their growth is stopped by mitomycin C treatment.

When the aforementioned feeder cells are not used in the cultivation of ES cells, the cultivation can be performed with the addition of an LIF (Leukemia Inhibitory Factor). As the LIF, mouse recombinant LIF, rat recombinant LIF (Nippon Chemi-Con Corporation and the like) and the like can be mentioned.

Although the number of days for the aforementioned culture conditions for ES cell is variable as appropriate depending on cell condition and the like, it is preferably about 1 day to 3 days.

When a gene comprising a drug resistance gene is used as the marker gene, selection with a medium comprising the corresponding drug (selection medium) is performed. The drug may be contained in the medium at the time of contact of a somatic cell and a test substance, and may be contained after

the contact. Furthermore, the aforementioned drug may be contained in the medium after cultivation under culture conditions for ES cell.

Following the aforementioned step, the presence or absence of the emergence of cells expressing the marker gene is determined, and a test substance allowing the emergence of the cells is elected as a somatic cell nuclear reprogramming substance candidate (step (b)). The step (b) is described below.

When the marker gene is a gene comprising a drug resistance gene, cells expressing the marker gene can be selected by cultivation using a selection medium as described above. Cells expressing the marker gene can be detected by observation using a fluorescence microscope when the marker gene is a fluorescent protein gene, by adding a luminescent substrate when the marker gene is a luminescent enzyme gene, and by adding a chromogenic substrate when the marker gene is a chromogenic enzyme gene.

If cells expressing the marker gene are detected compared with before addition of the test substance (including cases where the amount detected has increased), the test sample (test substance) used here is selected as a somatic cell nuclear reprogramming substance candidate.

The aforementioned screening can be repeatedly performed at any frequency as necessary. For example, when a mixture such as a cDNA library or a cell extract is used in the first screening, a somatic cell nuclear reprogramming factor candidate substance can finally be selected by repeatedly performing the same screening with the mixture divided (fractionated) in the second screening and beyond.

As an example of increasing the screening efficiency, a screening system wherein a test substance is added to fusion cells of somatic cells and ES cells is effective, rather than using the aforementioned somatic cells as is for the screening. Accordingly, the screening method of the present invention includes a screening method for a somatic cell nuclear

reprogramming substance comprising the following steps (a) and (b):

(a) a step for bringing into contact with each other a fusion cell (somatic cell) of a somatic cell comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene and an ES cell, and a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of the emergence of cells expressing the marker gene, and selecting a test substance allowing the emergence of the cells as a somatic cell nuclear reprogramming substance candidate,

"Fusion cells" as mentioned herein means fused cells of somatic cells and ES cells, wherein the aforementioned marker gene is not expressed (or is expressed at lower expression levels). If the number of colonies increases with the addition of a test substance compared with the number of ES-like-cell colonies resulting from fusion of somatic cells and ES cells, the test substance can be selected as a somatic cell nuclear reprogramming substance candidate.

As specific examples of the aforementioned screening method of the present invention, screening methods utilizing the ECAT2 gene, the ECAT3 gene, the ECAT4 gene and the ECAT5 gene, respectively, are described below; for all ECAT genes (ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene), screening can be performed in the same manner with reference to the following description.

Example 1: Screening utilizing the ECAT2 gene

As a specific example of the screening method of the present invention utilizing the ECAT2 gene, a screening method comprising the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT2 gene, and a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the emergence of the surviving cells as a somatic cell nuclear reprogramming substance candidate,
can be mentioned.

As shown in an Example below, the ECAT2 gene is not an essential factor for the maintenance and growth of ES cells. Therefore, it is preferable to perform the screening of the present invention using a somatic cell resulting from homozygously knocking in a marker gene to the ECAT2 gene.

A knock-in mouse wherein a marker gene has been homozygously knocked in to the ECAT2 gene ($ECAT2^{\beta geo/\beta geo}$ mouse) can be prepared by, for example, the method described in Example 3 below. Somatic cells such as lymphocytes and skin cells are isolated from this $ECAT2^{\beta geo/\beta geo}$ mouse. A test substance is added to these somatic cells, the cells are cultured under culture conditions for ES cell (see, for example, Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), and selected with G418 (0.25 mg/ml). If surviving cells are observed in the selection with G418, the test substance used here is selected as a somatic cell nuclear reprogramming substance candidate.

For example, when a cDNA library derived from ES cells is used as the test substance, a cDNA pool derived from a cDNA library is transfected to the aforementioned somatic cell by a known technique such as the lipofectin method, and selection with G418 is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the cDNA pool is further divided into some pools and transfected to somatic cells. By repeating this experiment, a somatic cell nuclear reprogramming factor (candidate) derived from ES cells can finally be selected.

Example 2: Screening utilizing the ECAT3 gene

As a specific example of the screening method of the

present invention utilizing the ECAT3 gene, a screening method comprising the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT3 gene, and a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the emergence of the surviving cells as a somatic cell nuclear reprogramming substance candidate,

can be mentioned.

As shown in an Example below, the ECAT3 gene is not an essential factor for the maintenance and growth of ES cells. Therefore, it is preferable to perform the screening of the present invention using a somatic cell wherein a marker gene has been homozygously knocked in to the ECAT3 gene.

A knock-in mouse wherein a marker gene has been knocked in to the ECAT3 gene ($ECAT3^{\beta geo/\beta geo}$ mouse) can be prepared by, for example, the method described in Example 1 below. Somatic cells such as lymphocytes and skin cells are isolated from this $ECAT3^{\beta geo/\beta geo}$ mouse. A test substance is added to these somatic cells, the cells are cultured under culture conditions for ES cell (see, for example, Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), and selected with G418 (0.25 mg/ml). If surviving cells are observed in the selection with G418, the test substance used here is selected as a somatic cell nuclear reprogramming substance candidate.

For example, when a cDNA library derived from ES cells is used as the test substance, a cDNA pool derived from a cDNA library is transfected to the aforementioned somatic cell by a known technique such as the lipofectin method, and selection with G418 is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the cDNA pool is further

divided into some pools and transfected to somatic cells. By repeating this experiment, a somatic cell nuclear reprogramming factor (candidate) derived from ES cells can finally be selected.

Example 3: Screening utilizing the ECAT4 gene

As a specific example of the screening method of the present invention utilizing the ECAT4 gene, a screening method comprising the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT4 gene, and a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the emergence of the surviving cells as a somatic cell nuclear reprogramming substance candidate,

can be mentioned.

The ECAT4 gene is an essential factor for the maintenance and growth of ES cells. Therefore, the screening of the present invention is performed using a somatic cell wherein a marker gene has been heterozygously knocked in to the ECAT4 gene.

A knock-in mouse wherein a marker gene has been heterozygously knocked in to the ECAT4 gene (ECAT4$^{\beta geo/+}$ mouse) can be prepared by the method described in the literature (Mitsui, K., et al., Cell, 113: 631-642 (2003)) and the like, and the following method, described briefly below, can be mentioned.

A targeting vector for replacing the exon 2 of the mouse ECAT4 gene with the IRES-β geo cassette (Mountford et al., Proc. Natl. Acad. Sci. USA, 91:4303-4307 (1994)) is prepared as described below. A 5′-side arm is prepared by amplifying a 4-kb fragment comprising the intron 1 of ECAT4 by PCR with mouse genomic DNA as the template using primers (AGGGTCTGCTACTGAGATGCTCTG (SEQ ID NO:39) and

AGGCAGGTCTTCAGAGGAAGGGCG (SEQ ID NO:40)). Also prepared is a 3'-side arm by amplifying a 1.5-kb fragment comprising exon 3-intron 3-exon 4 by PCR with mouse genomic DNA as the template using primers (CGGGCTGTAGACCTGTCTGCATTCTG (SEQ ID NO:41) and GGTCCTTCTGTCTCATCCTCGAGAGT (SEQ ID NO:42)). The 5'-side arm and the 3'-side arm are ligated to the IRES-β geo cassette to prepare a targeting vector. This targeting vector is cleaved with SacII and introduced by electroporation into RF8 ES cells (see Meiner et al., Proc. Natl. Acad. Sci USA, 93: 14041-14046 (1996)). Subsequently, a clone undergoing accurate homologous recombination is selected with a G418 selection medium. By injecting these ES cells undergoing homologous recombination with β geo into mouse blastocysts, a chimeric mouse is obtained, from which a heterozygous mutant mouse ($ECAT4^{\beta geo/+}$ mouse) is established.

Next, somatic cells such as lymphocytes and skin cells are isolated from this $ECAT4^{\beta geo/+}$ mouse. A test substance is added to these somatic cells, the cells are cultured under culture conditions for ES cell (see, for example, Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), and selected with G418 is performed. If surviving cells are observed in the selection with G418, the test substance used here is selected as a somatic cell nuclear reprogramming substance candidate.

For example, when a cDNA library derived from ES cells is used as the test substance, a cDNA pool derived from a cDNA library is transfected to the aforementioned somatic cell by a known technique such as the lipofectin method, and selection with G418 is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the cDNA pool is further divided into some pools and transfected to somatic cells. By repeating this experiment, a somatic cell nuclear reprogramming factor (candidate) derived from ES cells can finally be selected.

As another specific example of the aforementioned

screening method of the present invention utilizing the ECAT4 gene, a screening method comprising the following steps (a) and (b):

(a) a step for supplying ECAT4 to a somatic cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT4 gene, and bringing it into contact with a test substance,

(b) a step following the aforementioned step (a), for determining the presence of absence of surviving cells in a selection medium, and selecting a test substance allowing the emergence of the surviving cells as a somatic cell nuclear reprogramming substance candidate,

can be mentioned.

As described in the literature (Cell, 113: 631-642 (2003), WO 2004/067744), because ECAT4 is an essential factor for the maintenance of ES cell functions (undifferentiated state and pluripotency), a cell that is ECAT4 homozygous mutant ES cell, to which ECAT4 has been supplied, can be said to be a differentiated cell in a state similar to that of ES cells. Therefore, a screening system for bringing this cell into contact with a test substance is an efficient screening system enabling the easier identification of a nuclear reprogramming substance.

The ECAT4 homozygous mutant ES cell used here can be prepared by, for example, introducing the hygro vector (a targeting vector for replacing the ECAT4 gene with the Hygro vector) into the aforementioned ES cell undergoing homologous recombination with β geo (a heterozygous mutant cell wherein the β geo gene has been knocked in to the ECAT4 gene).

ECAT4 is supplied to this ECAT4 homozygous mutant ES cell (somatic cell). To effect the supply, an expression vector harboring the ECAT4 gene may be introduced into the cell and allowed to express, or the ECAT4 protein may be introduced in a form incorporatable into the cell (for example, in fusion with a protein like TAT).

At the same time as, or after, this introduction of ECAT4

(gene), a test substance is added, the cells are cultured under culture conditions for ES cell (see, for example, Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), and selected with G418 and/or hygromycin. If surviving cells are observed in the selection, the test substance used here is selected as a somatic cell nuclear reprogramming substance candidate.

For example, when a cDNA library derived from ES cells is used as the test substance, the ECAT4 gene is first introduced into the aforementioned somatic cell (ECAT4 homozygous mutant ES cell). Subsequently, a cDNA pool derived from a cDNA library is transfected by a known technique such as the lipofectin method, and selection with G418 and/or hygromycin is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the cDNA pool is further divided into some pools and transfected to somatic cells. By repeating this experiment, a somatic cell nuclear reprogramming factor (candidate) derived from ES cells can finally be selected.

Example 4: Screening utilizing the ECAT5 gene

As a specific example of the screening method of the present invention utilizing the ECAT5 gene, a screening method comprising the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT5 gene, and a test substance,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the emergence of the surviving cells as a somatic cell nuclear reprogramming substance candidate,

can be mentioned.

As shown in an Example below, the ECAT5 gene is not an essential factor for the maintenance of ES cells. Therefore, it is preferable to perform the screening of the present

invention using a somatic cell wherein a marker gene has been homozygously knocked in to the ECAT5 gene.

A knock-in mouse wherein a marker gene has been homozygously knocked in to the ECAT5 gene (ECAT5$^{\beta geo/\beta geo}$ mouse) can be prepared by, for example, the method described in Example 2 below (Japanese Patent Kokai Publication No. 2003-265166). Somatic cells such as lymphocytes and skin cells are isolated from this ECAT5$^{\beta geo/\beta geo}$ mouse. A test substance is added to these somatic cells, the cells are cultured under culture conditions for ES cell (see, for example, Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), and selected with G418 (0.25 mg/ml). If surviving cells are observed in the selection with G418, the test substance used here is selected as a somatic cell nuclear reprogramming substance candidate.

For example, when a cDNA library derived from ES cells is used as the test substance, a cDNA pool derived from a cDNA library is transfected to the aforementioned somatic cell by a known technique such as the lipofectin method, and selection with G418 is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the cDNA pool is further divided into some pools and transfected to somatic cells. By repeating this experiment, a somatic cell nuclear reprogramming factor (candidate) derived from ES cells can finally be selected.

Example 5: Screening utilizing two ECAT genes

As described above, a double knock-in mouse can be prepared by mating homozygous mutant mice wherein a marker gene has been knocked in to two different ECAT genes, and a somatic cell derived from the mouse can be used for the screening. Specifically, a screening method using a somatic cell derived from a double knock-in mouse concerning a combination of the ECAT2 gene and the ECAT3 gene can be mentioned as an example. As a specific example of the screening method of the present invention utilizing the ECAT2 gene and the ECAT3 gene, a

screening method comprising the following steps (a) and (b):
(a) a step for bringing into contact with each other a somatic cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to each of the ECAT2 gene and the ECAT3 gene, and a test substance,
(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the emergence of the surviving cells as a somatic cell nuclear reprogramming substance candidate,
can be mentioned.

It is desirable that the drug resistance genes knocked in here be different from each other between the ECAT2 gene and the ECAT3 gene. In this case, because double selection with two different drug resistance genes (for example, neomycin resistance gene and hygromycin resistance gene) is possible, the possibility of selecting false-positive ES-like cells in the screening of the present invention decreases, so that the likelihood of successful screening can be dramatically improved.

A double knock-in mouse of the ECAT2 gene and the ECAT3 gene ($ECAT2^{Hygro/Hygro}$ $ECAT3^{\beta geo/\beta geo}$ mouse) can be obtained by mating the $ECAT2^{Hygro/Hygro}$ mouse and $ECAT3^{\beta geo/\beta geo}$ mouse prepared in Examples 1 and 3 below (but the drug resistance gene is the hygromycin resistance gene). Somatic cells such as lymphocytes and skin cells are isolated from this $ECAT2^{Hygro/Hygro}$ $ECAT3^{\beta geo/\beta geo}$ mouse. A test substance is added to these somatic cells, the cells are cultured under culture conditions for ES cell (see, for example, Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), and selected with G418 (0.25 mg/ml) and hygromycin (0.1 mg/ml). If surviving cells are observed in this selection, the test substance used here is selected as a somatic cell nuclear reprogramming substance candidate.

For example, when a cDNA library derived from ES cells is used as the test substance, a cDNA pool derived from a cDNA

library is transfected to the aforementioned somatic cell by a known technique such as the lipofectin method, and selection with G418 and hygromycin is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the cDNA pool is further divided into some pools and transfected to somatic cells. By repeating this experiment, a somatic cell nuclear reprogramming factor (candidate) derived from ES cells can finally be selected.

Example 6: Screening using fusion cells

A test substance is added to the aforementioned fusion cells of the somatic cells of the present invention and ES cells, the fused cells are cultured under culture conditions for ES cell (see, for example, Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), and selected on the basis of the properties of a selection marker. If the number of colonies has increased with the addition of the test substance compared with the number of ES-like-cell colonies emerging as a result of fusion of somatic cells and ES cells, the test substance used here is selected as a somatic cell nuclear reprogramming substance candidate.

For example, when a cDNA library derived from ES cells is used as the test substance and a drug resistance gene is used as the marker, a cDNA pool derived from a cDNA library is transfected to the aforementioned fusion cell of somatic cells and ES cells by a known technique such as the lipofectin method, and selection with a drug is performed by the aforementioned technique to determine the number of surviving cells. If the number of surviving cells (number of ES-like-cell colonies) has increased compared with a system to which the test substance has not been added, the cDNA pool is further divided into some pools and transfected to fusion cells (or somatic cells before the fusion). By repeating this experiment, a somatic cell nuclear reprogramming factor (candidate) derived from ES cells can finally be selected.

Whether or not the somatic cell nuclear reprogramming

substance (candidate) selected by the screening of the present invention reprograms the nucleus of the somatic cell can be confirmed by determining (1) whether or not the ES-like-cell converted from a somatic cell by the nuclear reprogramming factor (candidate) is expressing an ES cell marker gene such as Oct3/4 or Ecat4 (Nanog), (2) whether or not the aforementioned ES cell differentiates in vitro with retinoic acid stimulation and the like, (3) whether or not a chimeric mouse is born after injection of the aforementioned ES cells into mouse blastocysts, and the like.

(2) Nuclear reprogramming substance of the present invention

The present invention provides a somatic cell nuclear reprogramming substance selected using the aforementioned screening method of the present invention. The nuclear reprogramming substance is a nucleic acid, a peptide, a protein, an organic compound, an inorganic compound or a mixture thereof. The ES cells used in Examples below are also among somatic cell nuclear reprogramming substances. Specifically, a gene or protein derived from ES cells can be mentioned as examples. As specific examples, a gene or protein derived from ES cells having the NAT1 gene destroyed can be mentioned. The nuclear reprogramming substance of the present invention is useful in stem cell therapy. Specifically, when somatic cells (tissue stem cells, differentiated cells and the like) are collected from a patient and the nuclear reprogramming substance of the present invention is added thereto, ES-like cells emerge. By allowing these ES-like cells to differentiate into nerve cells, myocardial cells or blood cells and the like using retinoic acid, growth factors (for example, EGF, FGF-2, BMP-2, LIF and the like), or glucocorticoid and the like, and returning these cells to the patient, stem cell therapy can be accomplished.

(3) New application for the knock-in mouse of the present invention (use as a source of the somatic cell for the screening of the present invention)

Traditionally, a knock-in mouse wherein a marker gene has

been knocked in to a gene has been utilized for functional analysis of the gene. In some cases, such a knock-in mouse has served as a disease model animal. However, there has been no utilization as a source of the somatic cell used in the new screening method disclosed herein.

The present invention provides an application for a knock-in mouse comprising a gene resulting from knocking in a marker gene to an ECAT gene as a source of the somatic cell used in the screening of the present invention.

Regarding the method of preparing the knock-in mouse and the like, the same as described in detail in "(1) Screening method of the present invention" above and Examples below applies. The knock-in mouse preferably homozygously comprises a gene resulting from knocking in a marker gene to the gene, when the ECAT2 gene, the ECAT3 gene and/or the ECAT5 gene is used. When a gene resulting from knocking in a marker gene to the ECAT4 gene is used, the knock-in mouse preferably heterozygously comprises such the gene. As the marker gene, a drug resistance gene, a fluorescent protein gene, a luminescent enzyme gene, a chromogenic enzyme gene or a gene comprising a combination thereof can be mentioned. A gene comprising a drug resistance gene is particularly preferable.

(4) Somatic cell of the present invention

The present invention provides a somatic cell comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene.

Regarding the method of preparing the somatic cell and the like, the same as described in detail in "(1) Screening method of the present invention for somatic cell nuclear reprogramming substance" above and Examples below applies. The somatic cell of the present invention is effectively used in the aforementioned screening method of the present invention or the ES-like cell selection method of the present invention described below.

(5) ES-like cell selection method of the present invention

The present invention also provides an ES-like cell selection method comprising the following steps (a) and (b):
(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene, and a somatic cell nuclear reprogramming substance,
(b) a step following the aforementioned step (a), for selecting cells expressing the marker gene as ES-like cells.

A somatic cell wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene as described with respect to the aforementioned screening method of the present invention is also effectively used for selecting ES-like cells. For example, it is desirable, with stem cell therapy in mind, that an ES-like cell emerging with stimulation of a human somatic cell with a nuclear reprogramming substance be separated (purified) from other cells (somatic cells), and used for subsequent treatment. Because the system of the present invention is a system enabling the easy selection of ES-like cells with the expression of a marker gene such as a drug resistance gene as the index, as described above, it can be effectively used in selecting and separating ES-like cells.

"ES-like cells" as mentioned herein means cells having ES cell properties, that is, cells having undifferentiated state and pluripotency.

The ES-like cell selection method of the present invention can be used for all purposes of selecting (separating) ES cells not only in the aforementioned treatment of humans, but also in various in vitro and in vivo studies concerning ES cells.

All of the aforementioned methods, namely 1) the method of preparing a somatic cell comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene, 2) the method of bringing into contact with each other the somatic cell and a

somatic cell nuclear reprogramming substance, and 3) the method of selecting cells expressing the marker gene, are the same as those described in "(1) Screening method of the present invention for somatic cell nuclear reprogramming substance". When a gene comprising a drug resistance gene as the marker gene is used, cells expressing the marker gene can easily be selected (separated) by cultivation in a selection medium. When a fluorescent protein gene, a luminescent enzyme gene, or a chromogenic enzyme gene is used as the marker gene, the cell can be selected (separated) by utilizing a cell sorter, the limiting dilution method or the soft agar colony method and the like.

"The nuclear reprogramming substance" as mentioned above refers to a substance involved in somatic cell nuclear reprogramming as obtained in the aforementioned screening of the present invention. In Examples below, cells expressing the marker gene are selected as ES-like cells using ES cells themselves as a somatic cell nuclear reprogramming substance.

In the ES-like cell selection method of the present invention, any ECAT gene (ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene) can be used. As a specific example, the following selection method can be mentioned.

Specifically, as the selection method utilizing the ECAT2 gene, an ES-like cell selection method comprising the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a drug resistance gene is present at a position permitting expression control by the expression control region of an ECAT2 gene, and a somatic cell nuclear reprogramming substance,

(b) a step following the aforementioned step (a), for selecting surviving cells in a selection medium as ES-like cells,

can be mentioned.

As the selection method utilizing the ECAT3 gene, an ES-like cell selection method comprising the following steps (a)

and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a drug resistance gene is present at a position permitting expression control by the expression control region of the ECAT3 gene, and a somatic cell nuclear reprogramming substance,

(b) a step following the aforementioned step (a), for selecting surviving cells in a selection medium as ES-like cells,

can be mentioned.

As the selection method utilizing the ECAT5 gene, an ES-like cell selection method comprising the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a drug resistance gene is present at a position permitting expression control by the expression control region of the ECAT5 gene, and a somatic cell nuclear reprogramming substance,

(b) a step following the aforementioned step (a), for selecting surviving cells in a selection medium as ES-like cells,

can be mentioned.

As the selection method utilizing the ECAT2 gene and the ECAT3 gene, an ES-like cell selection method comprising the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a drug resistance gene is present at positions permitting expression control by the expression control regions of the ECAT2 gene and the ECAT3 gene, and a somatic cell nuclear reprogramming substance,

(b) a step following the aforementioned step (a), for selecting surviving cells in a selection medium as ES-like cells,

can be mentioned.

As the selection method utilizing the ECAT4 gene, an ES-like cell selection method comprising the following steps (a) and (b):

(a) a step for bringing into contact with each other a somatic cell comprising a gene wherein a drug resistance gene is

present at a position permitting expression control by the expression control region of the ECAT4 gene, and a somatic cell nuclear reprogramming substance,

(b) a step following the aforementioned step (a), for selecting surviving cells in a selection medium as ES-like cells,

can be mentioned.

It is desirable, with treatment of humans in mind, that the somatic cell used in the ES-like cell selection method described above be a human somatic cell comprising a vector harboring a marker gene inserted at a position permitting expression control by the expression control region of an ECAT gene. Specifically, a somatic cell prepared as described below is used.

Specifically, first, somatic cells are prepared by isolating a patient somatic cell from a human and the like. As the somatic cell, somatic cells involved in disease, somatic cells involved in disease treatment and the like can be mentioned. Any vector described in section (1-2) above is introduced into this human somatic cell. Specifically, it is desirable that the BAC vector (BAC vector wherein a marker gene is present downstream of the expression control region of an ECAT gene) or the PAC vector be introduced. The BAC vector (PAC vector) introduced here may be one kind of BAC vector, and may be two or more kinds of BAC vectors comprising different ECAT genes. By adding a nuclear reprogramming substance to this BAC vector-incorporating cell, ES-like cells are allowed to emerge. These ES-like cells are selected depending on the properties of the marker gene used. For example, when a drug resistance gene is used as the marker gene, ES-like cells can easily be selected with the drug resistance as the index by selection with a selection medium after addition of a nuclear reprogramming substance.

(6) ES-like cells of the present invention

The present invention provides cells (ES-like cells) expressing a marker gene emerging by the screening of the present invention for a somatic cell nuclear reprogramming

substance, and ES-like cells selected by the ES-like cell selection method of the present invention. The ES-like cells can be effectively used in subsequent evaluations in vitro and in vivo. Specifically, examining the differentiation induction potential of the ES-like cells, the transplantation and survival of differentiation-induced cells to individuals (mouse and the like) and the like are of paramount importance in preliminary investigations of stem cell therapy in humans and various studies concerning ES cells. The ES-like cells of the present invention are effectively used in such studies and investigations.

Furthermore, by allowing the human cells expressing a marker gene (ES-like cells) obtained by the ES-like cell selection method of the present invention to differentiate into nerve cells, myocardial cells or blood cells and the like using retinoic acid, growth factors (for example, EGF, FGF-2, BMP-2, LIF and the like), or glucocorticoid and the like, and returning this to the patient, stem cell therapy can be achieved.

(6) Screening method of the present invention for substance for the maintenance of undifferentiated state and pluripotency of ES cells

The present invention provides a screening method for a substance for the maintenance of undifferentiated state and pluripotency of ES cells, which comprises the following steps (a) and (b):

(a) a step for bringing an ES cell comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene, into contact with a test substance in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells,

(b) a step following the aforementioned step (a), for determining the presence or absence of cells expressing the marker gene, and selecting a test substance allowing the occurrence of the cells as a candidate substance for the

maintenance of undifferentiated state and pluripotency of ES cells.

When ES cells wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene are cultured in a medium not allowing the maintenance of ES cell properties (undifferentiated state and pluripotency), the expression of the marker gene disappears. On the other hand, if a substance for the maintenance of undifferentiated state and pluripotency of ES cells is present in the aforementioned medium, the expression of the marker gene persists. By utilizing this property, a substance (candidate) for the maintenance of undifferentiated state and pluripotency of ES cells can easily be screened.

The ES cell used in the aforementioned screening step (a) may be any ES cell, as long as it comprises a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene. Specifically, for example, ES cells derived from the knock-in mouse described in (1-1-a) above, ES cells derived from the transgenic mouse described in (1-1-b) above, ES cells comprising the BAC vector or PAC vector described in (1-2-a) above, ES cells comprising the plasmid vector described in (1-2-b) above, or ES cells comprising the targeting vector described in (1-2-c) above can be mentioned. ES-like cells resulting from conversion of a somatic cell comprising a gene wherein a marker gene is present at a position permitting expression control by the expression control region of an ECAT gene as described above can also be used in the same way (hereinafter referred to as "ES cells", including ES-like cells).

"The medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells" used in the aforementioned screening step (a) may be any medium, as long as it is a medium not allowing the maintenance of ES cell properties or a medium not allowing the maintenance of

undifferentiated state. For example, because it is known that serum or feeder cells are essential for the maintenance of mouse ES cells (undifferentiated state and pluripotency maintenance) at low densities, the same conditions as the culture conditions for the ES cells, but deprived of serum or feeder cells or both, can be mentioned. Also, because feeder cells are essential for the maintenance of human ES cells (undifferentiated state and pluripotency maintenance), the same conditions as culture conditions for human ES cell, but deprived of feeder cells, can be mentioned. Furthermore, in the case of human ES cells, because cells that differentiate even in the presence of feeder cells emerge, the culture may be performed in the presence of feeder cells.

Specifically, the same conditions as the culture conditions for ES cell described in the literature (Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), but deprived of serum or feeder cells or both, and the like can be mentioned as examples.

The aforementioned step (a) is performed by bringing the aforementioned ES cell into contact with a test substance in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells. The test substance is brought into contact with the ES cell before, at, or after the ES cells are transferred to the medium not allowing the maintenance of undifferentiated state and pluripotency.

The test substance (test sample) used in this screening is not subject to limitation, and is exemplified by a nucleic acid, a peptide, a protein, an organic compound, an inorganic compound or a mixture thereof and the like; the screening of the present invention is specifically performed by bringing these test substances into contact with the aforementioned ES cell. As the test substance, a secretion product of cells, serum, a cell extract, a gene (genome, cDNA) library, an RNAi library, a nucleic acid (genome, cDNA, mRNA), an antisense nucleic acid, a low molecular compound, a high molecular compound, a protein, a peptide, a natural compound and the like

can be mentioned. Specifically, animal serum or a fraction thereof, a secretion product of feeder cells or a fraction thereof and the like can be mentioned.

These test substances (test samples) are brought into contact with somatic cells in an embodiment incorporatable into the somatic cells. For example, when the test substance is a nucleic acid (cDNA library and the like), it is introduced into somatic cells using calcium phosphate, DEAE-dextran, or a lipid for transfection.

When a gene comprising a drug resistance gene as the marker gene is used, selection is performed with a medium comprising the corresponding drug (selection medium). The drug may be contained in the medium at the time of contact of the ES cell and the test substance, and may be contained after the contact. Furthermore, the aforementioned drug may be contained in the medium after cultivation in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells in the presence of a test substance.

After the aforementioned step (a), the presence or absence of cells expressing the marker gene is determined, and a test substance allowing the occurrence of the cells is selected as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells (step (b)). Regarding the step (b), the same as described in "(1) Screening method of the present invention for somatic cell nuclear reprogramming substance" above applies. If cells expressing the marker gene are observed, the test sample (test substance) used here is selected as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells.

The aforementioned screening can be repeatedly performed at any frequency as necessary. For example, when a mixture such as a secretion product secreted of feeder cells or serum is used in the first screening, a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells can finally be selected by repeatedly performing the same

screening with the mixture divided (fractionated) in the second screening and beyond.

Note that when screening is performed using a mixture as the test sample as described above, a substance that promotes the growth of ES cells is possibly be selected along with a substance for the maintenance of undifferentiated state and pluripotency of ES cells. Specifically, when a mixture (fraction A) is subjected to the aforementioned screening method of the present invention, and if surviving cells are confirmed and the number of the surviving cells increases, it is considered that the fraction contains a substance that promotes the growth of ES cells along with a substance for the maintenance of undifferentiated state and pluripotency of ES cells (of course there are some cases wherein a single substance has the properties of the two substances). In that case, the fraction A is further fractionated; if surviving cells are observed but the number of cells does not increase when one resulting fraction (fraction B) is subjected to the screening of the present invention, and also if no surviving cells are observed when the other resulting fraction (fraction C) is subjected to the screening of the present invention, it is considered that the fraction B contains a substance for the maintenance of undifferentiated state and pluripotency of ES cells, whereas the fraction C contains a substance that promotes the growth of ES cells. The screening of the present invention is also useful in selecting such a substance (candidate) that promotes the growth of ES cells.

As specific examples of the aforementioned screening method, screening methods utilizing the ECAT2 gene, the ECAT3 gene, the ECAT4 gene and the ECAT5 gene, respectively, are described below; for all ECAT genes (ECAT1 gene, ECAT2 gene, ECAT3 gene, ECAT4 gene, ECAT5 gene, ECAT6 gene, ECAT7 gene, ECAT8 gene, ECAT9 gene and Oct3/4 gene), screening can be performed in the same manner with reference to the following description.

Example 1: Screening utilizing the ECAT2 gene

As a specific example of the screening method for a substance for the maintenance of undifferentiated state and pluripotency of ES cells utilizing the ECAT2 gene, a screening method comprising the following steps (a) and (b):

(a) a step for bringing an ES cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT2 gene into contact with a test substance in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the occurrence of the surviving cells as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells,

can be mentioned.

As shown in an Example below, the ECAT2 gene is not an essential factor for the maintenance and growth of ES cells. Therefore, it is preferable to perform the screening of the present invention using ES cells wherein a marker gene has been homozygously knocked in to the ECAT2 gene. The ES cells can be prepared by, for example, the method described in Example 3 (ECAT2 gene homozygous mutant RF8 ES cell). These ES cells are cultured in the presence of a test substance under the same conditions as the culture conditions for ES cell described in the literature (Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), but deprived of serum or feeder cells or both. Subsequently, selection with G418 and/or hygromycin is performed. If surviving cells are observed in the selection with these drugs, the test substance used here is selected as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells.

For example, when a secretion product of feeder cells is used as the test substance, the secretion product of feeder cells is added to the aforementioned ES cells, and selection with G418 and/or hygromycin is performed by the aforementioned

technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the secretion product is further divided into some fractions and added to ES cells. By repeating this experiment, a factor (candidate) for the maintenance of undifferentiated state and pluripotency of ES cells can finally be selected. Additionally, a substance (candidate) that promotes the growth of ES cells can also be selected with the increase in the number of viable cells as the index.

Example 2: Screening utilizing the ECAT3 gene

As a specific example of the screening method for a substance for the maintenance of undifferentiated state and pluripotency of ES cells utilizing the ECAT3 gene, a screening method comprising the following steps (a) and (b):

(a) a step for bringing an ES cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT3 gene into contact with a test substance in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the occurrence of the surviving cells as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells,

can be mentioned.

As shown in an Example below, the ECAT3 gene is not an essential factor for the maintenance and growth of ES cells. Therefore, it is preferable to perform the screening of the present invention using ES cells wherein a marker gene has been homozygously knocked in to the ECAT3 gene. Using these ES cells, ES cells wherein the ECAT3 gene has been homozygously mutated can be prepared by, for example, further introducing the Hygro vector (a targeting vector for replacing the ECAT3 gene with the Hygro gene) into the ES cells undergoing homologous recombination with the β geo vector, prepared in

Example 1. These cells are cultured in the presence of a test substance under the same conditions as the ES cell culturing conditions described in the literature (Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), but deprived of serum or feeder cells or both. Subsequently, selection with G418 and/or hygromycin is performed. If surviving cells are observed in the selection with these drugs, the test substance used here is selected as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells.

For example, when a secretion product of feeder cells is used as the test substance, the secretion product of feeder cells is added to the aforementioned ES cells, and selection with G418 and/or hygromycin is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the secretion product is further divided into some fractions and added to ES cells. By repeating this experiment, a factor (candidate) for the maintenance of undifferentiated state and pluripotency of ES cells can finally be selected. Additionally, a substance (candidate) that promotes the growth of ES cells can also be selected with the increase in the number of viable cells as the index.

Example 3: Screening utilizing the ECAT4 gene

As a specific example of the screening method for a substance for the maintenance of undifferentiated state and pluripotency of ES cells utilizing the ECAT4 gene, a screening method comprising the following steps (a) and (b):

(a) a step for bringing an ES cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT4 gene into contact with a test substance in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the

occurrence of the surviving cells as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells,

can be mentioned.

The ECAT gene is an essential factor for the maintenance and growth of ES cells. Therefore, it is preferable to perform the screening of the present invention using ES cells wherein a marker gene has been heterozygously knocked in to the ECAT4 gene.

ES cells wherein a marker gene has been heterozygously knocked in to the ECAT4 gene can be prepared by introducing a targeting vector (for example, a targeting vector for replacing the ECAT4 gene with the β geo gene) into ES cells to cause homologous recombination as in the aforementioned cases of ECAT2 and ECAT3. These cells are cultured in the presence of a test substance under the same conditions as the culture conditions for ES cell described in the literature (Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), but deprived of serum or feeder cells or both. Subsequently, selection with G418 is performed. If surviving cells are observed in the selection with G418, the test substance used here is selected as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells.

For example, when a secretion product of feeder cells is used as the test substance, the secretion product of feeder cells is added to the aforementioned ES cells, and selection with G418 is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the secretion product is further divided into some fractions and added to ES cells. By repeating this experiment, a factor (candidate) for the maintenance of undifferentiated state and pluripotency of ES cells can finally be selected. Additionally, a substance (candidate) that promotes the growth of ES cells can also be selected with the increase in the number of viable cells as an

index.

Example 4: Screening utilizing the ECAT5 gene

As a specific example of the screening method for a substance for the maintenance of undifferentiated state and pluripotency of ES cells utilizing the ECAT5 gene, a screening method comprising the following steps (a) and (b):

(a) a step for bringing an ES cell comprising a gene resulting from knocking in a gene comprising a drug resistance gene to the ECAT5 gene into contact with a test substance in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells,

(b) a step following the aforementioned step (a), for determining the presence or absence of surviving cells in a selection medium, and selecting a test substance allowing the occurrence of the surviving cells as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells,

can be mentioned.

As shown in an Example below, the ECAT5 gene is not an essential factor for the maintenance of ES cells. Therefore, it is preferable to perform the screening of the present invention using ES cells wherein a marker gene has been homozygously knocked in to the ECAT5 gene. The method of preparing the ES cell and the screening method using the same are the same as in the aforementioned cases of ECAT2 and ECAT3.

Whether or not the substance (candidate) for maintenance of undifferentiated state and pluripotency of ES cells selected by the aforementioned screening of the present invention maintains undifferentiated state and pluripotency of ES cells can be confirmed by culturing ES cells in a medium not allowing the maintenance of undifferentiated state and pluripotency of ES cells under culture conditions with the addition of the candidate substance, and examining various potentials thereof as ES cells. Specifically, this can be confirmed by, for example, determining (1) whether or not the ES cells cultured under the aforementioned culture conditions are expressing an

ES cell marker gene such as Oct3/4 or Ecat4 (Nanog), (2) whether or not the aforementioned ES cells differentiate in vitro with retinoic acid stimulation and the like, (3) whether or not a chimeric mouse is born after injection of the aforementioned ES cells to mouse blastocysts, and the like.

(7) Substance of the present invention for the maintenance of undifferentiated state and pluripotency of ES cells

The present invention provides a substance for the maintenance of undifferentiated state and pluripotency of ES cells selected using the aforementioned screening method. The substance for the maintenance of undifferentiated state and pluripotency of ES cells is any of a nucleic acid, a peptide, a protein, an organic compound, and an inorganic compound, and is preferably exemplified by a secretion product of feeder cells or a serum-derived component. The substance of the present invention for the maintenance of undifferentiated state and pluripotency of ES cells is useful in the clinical application of ES cells. Specifically, since it is essential to culture human ES cells or differentiated cells differentiated therefrom in a serum-free medium in the absence of feeder cells in clinical application, clinical application of the aforementioned ES cells is made possible by adding the substance of the present invention for the maintenance of undifferentiated state and pluripotency of ES cells to a serum-free medium.

(8) New application for the knock-in mouse of the present invention (use as a source of the ES cell for the screening of the present invention)

The present invention provides an application for the knock-in mouse of the present invention as a source of the ES cell for screening for a substance for the maintenance of undifferentiated state and pluripotency of ES cells. The knock-in mouse of the present invention is as described in (3) above. Isolation of ES cells from a knock-in mouse can be performed by a technique well known to those skilled in the art.

(9) ES cell of the present invention

The present invention provides an ES cell comprising a gene wherein a marker gene is allowed to be present at a position permitting expression control by the expression control region of an ECAT gene. The method of preparing the ES cell and the like are as described in detail in (1) and (6) above. The ES cell of the present invention is effectively used in a screening method for a substance for the maintenance of undifferentiated state and pluripotency of ES cells.

Examples

The present invention is hereinafter described specifically by means of the following Examples, which, however, are not to be construed as limiting the scope of the present invention.

Example 1

ES-like cell selection system utilizing the ECAT3 gene

A homozygous mutant knock-in mouse wherein the coding region of the ECAT3 gene had been replaced with the fusion gene of the β galactosidase and neomycin resistance genes (β geo) to knock out the ECAT3 gene, and wherein the expression of the ECAT3 gene had been made to permit monitoring by X-Gal staining and drug resistance (hereinafter $ECAT3^{\beta geo/\beta geo}$ mouse), was prepared. This $ECAT3^{\beta geo/\beta geo}$ mouse was prepared on the basis of the description in the literature (Tokuzawa, Y., et al., Molecular and Cellular Biology, 23(8): 2699-2708 (2003)). The procedure is briefly described below.

First, the BAC clone comprising the mouse ECAT3 gene was identified from a DNA pool of the BAC library (Research Genetics) by PCR screening using a portion of ECAT3 cDNA as the primer, and the base sequence thereof was determined.

Targeting vectors for replacing the exon 3 to exon 7 of the mouse ECAT3 gene with the IRES-β geo cassette (Mountford et al., Proc. Natl. Acad. Sci. USA, 91:4303-4307 (1994)) were prepared as described below. A 5′-side arm was prepared by amplifying a 1.4-kb fragment comprising the intron 1 to exon 3

of ECAT3 by PCR with the aforementioned mouse BAC DNA as the template using primers (ACCAAGGTCACCGCATCCAA (SEQ ID NO:43) and CTTCACCAAGATTTCCGATG (SEQ ID NO:44)). Also prepared was a 3'-side arm by amplifying a 3.5-kb fragment comprising exon 7 to exon 8 by PCR with mouse BAC DNA as the template using primers (GAATGGTGGACTAGCTTTTG (SEQ ID NO:45) and TGCCATGAATGTCGATATGCAG (SEQ ID NO:46)). The 5'-side arm and the 3'-side arm were ligated to the β geo cassette to prepare a targeting vector. This targeting vector was cleaved with NotI and introduced by electroporation into RF8 ES cells (Meiner et al., Proc. Natl. Acad. Sci USA, 93: 14041-14046 (1996)). A clone undergoing accurate homologous recombination was selected using a G418 selection medium. By injecting these ES cells undergoing homologous recombination with β geo into mouse (C57BL/6) blastocysts, a chimeric mouse was prepared, from which a heterozygous mutant mouse ($ECAT3^{\beta geo/+}$ mouse) was established; when such heterozygous mutant mice were mated, a homozygous mutant mouse ($ECAT3^{\beta geo/\beta geo}$ mouse) was born in accordance with Mendel's law.

Next, lymphocytes were collected from the thymus of an $ECAT3^{\beta geo/\beta geo}$ mouse by a conventional method. These cells were cultured under the culture conditions for ES cell described in the literature (Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)) for 2 days, and selection with G418 (0.25 mg/ml) was performed. As a result, all these lymphocytes died, with absolutely no drug resistant colony obtained. It was also confirmed that all normal ES cells died at this G418 concentration.

Next, lymphocytes derived from an $ECAT3^{\beta geo/\beta geo}$ mouse and RF8 cells were electrically fused in accordance with the method of Tada et al. (Tada, M., et al., Curr. Biol., 11(19): p1553-1558 (2001)), the resulting fusion cells were cultured on feeder cells (STO cells) under the aforementioned culture conditions for ES cell for 2 days, and selection was performed with G418 (0.25 mg/ml); a large number of ES-cell-like colonies were obtained. These colonies were isolated and cultured, and

RNA was recovered. Because Northern blotting revealed that these cells expressed Oct3/4 or ECAT4 (Nanog) in all clones, and also because transplantation of these clones to mouse blastocysts resulted in the formation of a chimeric mouse, it was demonstrated that the cells selected with G418 were ES-like cells surely having ES cell properties (Figure 1). Analysis of these cells by flow cytometry (FACS) showed that the size (Forward scatter) about doubled and the DNA content quadrupled (Figure 2). From these results, it was found that these colonies had become resistant to G418 because lymphocyte nuclear reprogramming (conversion to ES cells) occurred as a result of fusion of lymphocytes derived from an ECAT3$^{\beta geo/\beta geo}$ mouse and normal ES cells. Hence, somatic cells derived from the ECAT3$^{\beta geo/\beta geo}$ mouse become drug-resistant only when converted to ES-like cells. Therefore, it was demonstrated that by utilizing this property, ES-like cells can be selected and a nuclear reprogramming factor that induces conversion to ES-like cells can easily be screened.

Example 2

ES-like cell selection system utilizing the ECAT5 gene

A homozygous mutant knock-in mouse wherein the coding region of the ECAT5 gene had been replaced with β geo (ECAT5$^{\beta geo/\beta geo}$ mouse) was prepared on the basis of a method described in the literature (Takahashi, K., K. Mitsui, and S. Yamanaka, Nature, 423(6939): p541-545 (2003), Japanese Patent Unexamined Publication No. 2003-265166). Experiments were performed with the same protocol as described above using lymphocytes derived from this ECAT5$^{\beta geo/\beta geo}$ mouse. When $2x10^6$ lymphocytes from the ECAT5$^{\beta geo/\beta geo}$ mouse were fused with $4x10^5$ ES cells and selection culture with G418 was performed, similar ES-cell-like colonies were obtained, though the number thereof was smaller than that obtained in the case of ECAT3 in Example 1. Hence, it was found that ECAT5 could likewise be utilized in an ES-like cell selection system.

Regarding the reason for the smaller number of colonies compared with the case of ECAT3, it was considered that because

ECAT3 is not essential for the maintenance and growth of ES cells, whereas ECAT5 is a factor that promotes the growth of ES cells, despite the fact that the two share the feature of highly specific expression in ES cells, the reduction in the amount of the ECAT5 gene (knockout) serves unfavorably for conversion to ES cells.

Example 3

ES-like cell selection system utilizing the ECAT2 gene

Specific expression of the ECAT2 gene in ES cells has already been shown by Northern blot analysis (see International Patent Publication No. WO 02/097090). Further extensive expressional analysis by RT-PCR confirmed specific expression in undifferentiated ES cells (Figure 3A). When the cycle number was increased, expression occurred in the testis and ovary but absolutely no expression was observed in somatic tissue (Figure 3B).

The mouse ECAT2 genome sequence was identified by the public database Mouse Genome Resources (http://www.ncbi.nlm.nih.gov/genome/guide/mouse/). A BAC clone comprising this ECAT2 genome was cloned by PCR and Southern hybridization.

A targeting vector for replacing exons 1 to 3 with β geo (fusion gene of the β galactosidase and neomycin resistance genes) or Hygro (hygromycin resistance gene) was prepared to knock out the ECAT2 gene. Specifically, a targeting vector designed to replace the exons 1 to 3 of the mouse ECAT2 gene with the IRES (internal ribosome entry site)-β geo cassette or the IRES-Hygro cassette was prepared.

Specifically, first, a fragment comprising the 5′ flanking region to exon 1 region of the mouse ECAT2 genome and a fragment comprising the exon 3 to 3′ flanking region were each amplified by PCR with the aforementioned BAC clone as the template, and these were used as the 5′-arm and 3′-arm, respectively, of the targeting vector. The 5′-arm was amplified using primers (CCGCGGAAAGTCAAGAGATTGGGTGG (SEQ ID NO:47) and GCGGCCGCCTTTACGGGTCACGAGGGTCAC (SEQ ID NO:48)), and

the 3′-arm was amplified using primers (TGTGGCCAGTGTTTGGTTCTGGCGGG (SEQ ID NO:49) and CTCGAGGACTCGCCATTCTAGCCAAG (SEQ ID NO:50)). By ligating the two amplified fragments to the IRES-β geo cassette or IRES-Hygro cassette of pBSSK(-)-IRES-β geo or pBSSK(-)-IRES-Hygro, a targeting vector was developed, and this was linearized by cleavage with SacII.

An outline of the destruction of the ECAT2 gene with the aforementioned targeting vector is shown in Figure 4.

The linearized targeting vector was introduced into RF8 ES cells (Meiner, V. et al., Proc. Natl. Acad. Sci. USA, 93: 14041-14046 (1996)) by electroporation, and selection was performed with each drug (neomycin (G418) for β geo, hygromycin for Hygro). Accurate occurrence of homologous recombination was confirmed by Southern blotting. Specifically, genomic DNA extracted from the aforementioned ES cell was cleaved with PstI, after which it was electrophoresed and transferred onto a nylon membrane. This was hybridized with the 3′ region probe of the ECAT2 gene. An 18-kbp band is detected as emerging from the normal genome, a 13-kbp band is detected in homologous recombination with the β geo vector, and a 9-kbp band is detected in homologous recombination with the Hygro vector. The results are shown in Figure 5. Accurate homologous recombination in each drug resistance ES cell was confirmed.

Furthermore, when the β geo vector was introduced into ES cells undergoing homologous recombination with the Hygro vector and selection with neomycin was performed, three clones of ES cells wherein homologous recombination with both vectors occurred, hence the ECAT2 gene were homozygously mutated. Accurate occurrence of homologous recombination with both the β geo vector and the Hygro vector was confirmed by Southern blotting in the same manner as described above (Figure 5). Also, Northern blotting confirmed that these clones had lost the expression of ECAT2 (Figure 6).

As a result of an examination to determine whether or not these homozygous mutant ES cells maintained ES cell functions,

the cells were found to be normal in all of morphology, growth, and differentiation potential. From the results above, ECAT2 was found to be a factor that is specifically expressed in ES cells, testis, and ovary, but is not essential for the maintenance and initial development of ES. Thus, it was demonstrated that ECAT2, like the ECAT3 gene, could be highly effectively utilized for the selection of ES cells.

Next, by injecting ES cells undergoing homologous recombination with β geo into mouse (C57BL/6) blastocysts, a chimeric mouse was obtained, from which a heterozygous mutant mouse was established. Furthermore, when such heterozygous mutant mice were mated, a homozygous mutant mouse was born in accordance with Mendel's law. By performing experiments with the same protocol as Example 1 using somatic cells derived from this homozygous mutant mouse, ES-cell-like colonies can be obtained in the same manner as Example 1.

Specifically, when lymphocytes were collected from the thymus of an $ECAT2^{\beta geo/\beta geo}$ mouse by a conventional method, these lymphocytes and ES cells (RF8 cells) were fused using the same protocol as Example 1, and selection culture with G418 was performed, a large number of ES-cell-like colonies were obtained as in Example 1. Hence, it was found that ECAT2, like ECAT3, could be utilized for screening for a nuclear reprogramming factor and the like.

Example 4

Screening for somatic cell nuclear reprogramming substance using ECAT4 homozygous mutant ES cells

ES cells wherein the ECAT4 gene had been homozygously mutated (RF8 ES cells wherein the ECAT4 gene had been knocked in with both the β geo vector and the Hygro vector) were prepared on the basis of the literature (Mitsui, K., et al., Cell, 113: 631-642 (2003)) and WO 2004/067744). These ECAT4 homozygous mutant ES cells are known to no longer maintain undifferentiated state and pluripotency, hence to have differentiated (Cell, 113: 631-642 (2003), WO 2004/067744). When these cells were infected with a retroviral vector

comprising the ECAT4 gene and allowed to normally express ECAT4 therein, ES cell functions (undifferentiated state and pluripotency) were not restored. From this result, it was demonstrated that nuclear reprogramming of differentiated ES cells could not be performed with ECAT4 alone.

Because ECAT4 is an essential factor for the maintenance of ES cell functions (undifferentiated state and pluripotency), as described in the literature (Cell, 113: 631-642 (2003), WO 2004/067744), the aforementioned ES cell, wherein ECAT4 has been knocked out and ECAT4 has been supplied, can be said to be a differentiated cell in a state similar to that of ES cells. Therefore, a screening system for bringing this cell into contact with a test substance was considered to be an efficient screening system enabling the easier identification of a nuclear reprogramming substance.

Screening for a somatic cell nuclear reprogramming substance using the aforementioned ECAT4 homozygous mutant ES cells is performed as described below.

First, an ECAT4 gene expression vector is introduced into the aforementioned ECAT4 homozygous mutant ES cell to supply ECAT4 to the cell. Next, a test substance is added, cell culture is performed under culture conditions for ES cell (see, for example, Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), and selection with G418 and/or hygromycin is performed. If surviving cells are observed in the selection, the test substance used here is selected as a somatic cell nuclear reprogramming substance candidate.

For example, when a cDNA library derived from ES cells is used as the test substance, the ECAT4 gene is first introduced into the aforementioned somatic cell (ECAT4 homozygous mutant ES cell). Subsequently, a cDNA pool derived from a cDNA library is transfected by a known technique such as the lipofectin method, and selection with G418 and/or hygromycin is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the cDNA pool is further divided into some pools

and transfected to somatic cells. By repeating this experiment, a somatic cell nuclear reprogramming factor (candidate) derived from ES cells can finally be selected.

Example 5

cDNA library as a source for search of somatic cell nuclear reprogramming factor

NAT1 gene knockout ES cells were prepared on the basis of the literature (Yamanaka, S. et al., Embo J., 19, 5533-5541 (2000). These ES cells are resistant to G418 because they were prepared using a targeting vector harboring the neomycin resistance gene. However, utilizing the fact that the neomycin resistance gene used is surrounded by two LoxP sequences, the neomycin resistance gene was removed by allowing the expression of the CRE gene by the same cells to establish NAT1 gene knockout ES cells having again become sensitive to G418. As a result of cell fusion of these cells with thymocytes derived from an ECAT3 knock-in mouse, no remarkable difference in fusion efficiency was observed compared with normal ES cells (Figures 7 and 8). However, the frequency of the emergence of ES-cell-like colonies after the selection with G418 increased significantly compared with the use of normal ES cells (Figure 9). These results showed that the NAT1 gene knockout ES cells are higher than normal ES cells in terms of not only the degree of undifferentiated state, but also nuclear reprogramming activity, and are effective as a derivation for the cDNA library used for functional cloning of a nuclear reprogramming factor.

A cDNA library is constructed from NAT1 gene knockout ES cells using a commercially available cDNA library construction kit. Next, a cDNA pool derived from the aforementioned cDNA library is transfected to somatic cells derived from an ECAT3$^{\beta geo/\beta geo}$ mouse, an ECAT2$^{\beta geo/\beta geo}$ mouse and the like by a known technique such as the lipofectin method, and selection with G418 is performed to confirm the presence or absence of surviving cells. If surviving cells are identified, the cDNA pool is further divided into some pools and transfected to

somatic cells. By repeating this experiment, a somatic cell nuclear reprogramming factor (candidate) derived from ES cells can finally be selected.

Example 6

Screening for somatic cell nuclear reprogramming substance using somatic cells derived from ECAT3$^{\beta geo/\beta geo}$ mouse

Somatic cells such as lymphocytes and skin cells are isolated from an ECAT3$^{\beta geo/\beta geo}$ mouse. A test substance is added to these somatic cells, cell culture is performed under the culture conditions for ES cell described in the literature (Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)) and the like, and selection with G418 (0.25 mg/ml) is performed. If surviving cells are observed in the selection with G418, the test substance used here is selected as a somatic cell nuclear reprogramming substance candidate.

For example, when a cDNA library derived from ES cells is used as the test substance, a cDNA pool derived from a cDNA library is transfected to the aforementioned somatic cell by a known technique such as the lipofectin method, and selection with G418 is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the cDNA pool is further divided into some pools and transfected to somatic cells. By repeating this experiment, a somatic cell nuclear reprogramming factor (candidate) derived from ES cells can finally be selected.

Example 7

Screening for somatic cell nuclear reprogramming substance using somatic cells derived from ECAT2$^{\beta geo/\beta geo}$ mouse

Somatic cells such as lymphocytes and dermal cells are isolated from an ECAT2$^{\beta geo/\beta geo}$ mouse. A test substance is added to these somatic cells, cell culture is performed under the culture conditions for ES cell described in the literature (Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)) and the like, and selection with G418

(0.25 mg/ml) is performed. If surviving cells are observed in the selection with G418, the test substance used here is selected as a somatic cell nuclear reprogramming substance candidate.

For example, when a cDNA library derived from ES cells is used as the test substance, a cDNA pool derived from a cDNA library is transfected to the aforementioned somatic cell by a known technique such as the lipofectin method, and selection with G418 is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the cDNA pool is further divided into some pools and transfected to somatic cells. By repeating this experiment, a somatic cell nuclear reprogramming factor (candidate) derived from ES cells can finally be selected.

Example 8

Screening for somatic cell nuclear reprogramming substance using somatic cells derived from $ECAT2^{Hygro/Hygro}$-$ECAT3^{\beta geo/\beta geo}$ double knock-in mouse

An $ECAT2^{Hygro/Hygro}$-$ECAT3^{\beta geo/\beta geo}$ double knock-in mouse can be obtained by mating an $ECAT2^{Hygro/Hygro}$ mouse and an $ECAT3^{\beta geo/\beta geo}$ mouse. Somatic cells such as lymphocytes and skin cells are isolated from this double knock-in mouse. A test substance is added to these somatic cells, cell culture is performed under the culture conditions for ES cell described in the literature (Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)) and the like, and selection with G418 (0.25 mg/ml) and hygromycin (0.1 mg/ml) is performed. If surviving cells are observed in the selection with the two drugs, the test substance used here is selected as a somatic cell nuclear reprogramming substance candidate.

For example, when a cDNA library derived from ES cells is used as the test substance, a cDNA pool derived from a cDNA library is transfected to the aforementioned somatic cell by a known technique such as the lipofectin method, and selection with a drug is performed by the aforementioned technique to

confirm the presence or absence of surviving cells. If surviving cells are identified, the cDNA pool is further divided into some pools and transfected to somatic cells. By repeating this experiment, a somatic cell nuclear reprogramming factor (candidate) derived from ES cells can finally be selected.

Example 9

Screening for substance for the maintenance of undifferentiated state and pluripotency of ES cells using ECAT2 gene homozygous mutant ES cells

RF8 ES cells wherein the ECAT2 gene has been homozygously mutated, prepared in Example 3, are cultured in the presence of a test substance under the same conditions as the culture conditions for ES cell described in the literature (Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), but deprived of serum or feeder cells or both. Subsequently, selection with G418 (0.25 mg/ml) and/or hygromycin (0.1 mg/ml) is performed. If surviving cells are observed in the selection with these drugs, the test substance used here is selected as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells.

For example, when a secretion product of feeder cells is used as the test substance, the secretion product of feeder cells is added to the aforementioned ES cells, and selection with G418 and/or hygromycin is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the secretion product is further divided into some fractions and added to ES cells. By repeating this experiment, a factor (candidate) for the maintenance of undifferentiated state and pluripotency of ES cells can finally be selected.

Example 10

Screening for substance for the maintenance of undifferentiated state and pluripotency of ES cells using ECAT3 gene homozygous mutant ES cells

The Hygro vector (a targeting vector for replacing the ECAT3 gene with the Hygro gene) is introduced into ES cells undergoing homologous recombination with the β geo vector, prepared in Example 1, to prepare RF8 ES cells wherein the ECAT3 gene has been homozygously mutated. These cells are cultured in the presence of a test substance under the same conditions as the culture conditions for ES cell described in the literature (Meiner, V.L., et al., Proc. Natl. Acad. Sci. USA, 93(24): p14041-14046 (1996)), but deprived of serum or feeder cells or both. Subsequently, selection with G418 (0.25 mg/ml) and/or hygromycin (0.1 mg/ml) is performed. If surviving cells are observed in the selection with these drugs, the test substance used here is selected as a candidate substance for the maintenance of undifferentiated state and pluripotency of ES cells.

For example, when a secretion product of feeder cells is used as the test substance, the secretion product of feeder cells is added to the aforementioned ES cells, and selection with G418 and/or hygromycin is performed by the aforementioned technique to confirm the presence or absence of surviving cells. If surviving cells are identified, the secretion product is further divided into some fractions and added to ES cells. By repeating this experiment, a factor (candidate) for the maintenance of undifferentiated state and pluripotency of ES cells can finally be selected.

Industrial Applicability

According to the present invention, an efficient screening method for a somatic cell nuclear reprogramming substance is provided. Nuclear reprogramming substances are substances of paramount importance for realizing stem cell therapy; the screening method of the present invention enables early detection of such nuclear reprogramming substances. Furthermore, according to the present invention, an efficient screening method for substances for the maintenance of undifferentiated state and pluripotency of ES cells is

provided. Substances for the maintenance of undifferentiated state and pluripotency of ES cells are substances of paramount importance for clinical application of ES cells; the screening method of the present invention enables early detection of such substances for the maintenance of undifferentiated state and pluripotency of ES cells.

Sequence Listing Free Text

The base sequence shown in SEQ ID NO:39 is a primer.
The base sequence shown in SEQ ID NO:40 is a primer.
The base sequence shown in SEQ ID NO:41 is a primer.
The base sequence shown in SEQ ID NO:42 is a primer.
The base sequence shown in SEQ ID NO:43 is a primer.
The base sequence shown in SEQ ID NO:44 is a primer.
The base sequence shown in SEQ ID NO:45 is a primer.
The base sequence shown in SEQ ID NO:46 is a primer.
The base sequence shown in SEQ ID NO:47 is a primer.
The base sequence shown in SEQ ID NO:48 is a primer.
The base sequence shown in SEQ ID NO:49 is a primer.
The base sequence shown in SEQ ID NO:50 is a primer.